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A Quarterly Journal of Zoölogical Research

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A STUDY OF THE RATE OF OXYGEN CONSUMPTION OF DIFFERENT CLADOCERA CLONES DERIVED ORIGINALLY FROM A SINGLE MOTHER

V. OBRESHKOVE AND A. M. BANTA

St. Stephen's College, Columbia University, and the Department of Genetics of the Carnegie Institution of Washington

IN THE laboratory of the Department of Genetics of the Carnegie Institution of Washington, have been reared several groups of parthenogenetic strains ("pure lines" or more strictly "clones") of Cladocera descended from a single common mother. These separate clones have been bred for many generations, each as a separate individual parthenogenetic pedigree. In the case of certain individual clones of these groups, morphological mutations have occurred; and in certain other instances obvious physiological differences in reproductive characteristics have appeared. Both the new morphological characteristics and the new physiological characteristics, once they appear, are continued from generation to generation in parthenogenesis, i.e., are inherited. Some of the morphologically changed characters in *Daphnia longispina* have been followed through sexual reproduction. They proved to be simple dominant Mendelian characters (Banta and Wood, 1928*b*). One of these characters is sex-intergradedness. Its inheritance has been followed through a large number of generations in parthenogenesis. In sexual reproduction when individuals from the sex-intergrade strains are mated with individuals of non-sex-intergrade strains, half the sexually produced young bear sex-intergradedness. Hence this char-

acteristic clearly appears to be the expression of a dominant Mendelian factor which is heterozygous in the original sex-intergrade stock.

One of the groups of strains or clones studied in parthenogenesis consists of a number of strains derived from one of our original strains (Line 740) of *Simocephalus exspinosus*. In October, 1917, sex-intergrades were found in part of this strain and then, and subsequently, substrains were isolated and bred parthenogenetically for long periods of time. Three of these clones provided the material for this study. One of these clones (Strain II) derived from Line 740 was a normal, i.e., did not bear the sex-intergrade character. The others (Strains V and XVI) carried the sex-intergrade character but were very low for this character, i.e., sex-intergradedness was very slightly manifest. Differences in reproductive characteristics were apparent between Strain II and Strains V and XVI, Strain II being much the more vigorous. The present study was undertaken to learn if between these strains there were differences, perhaps corresponding with differences in reproductive vigor, in rate of oxygen consumption between intergrade and non-intergrade strains. But, while two of the strains used in this study were very slightly intergrade, no other strains of sex-intergrades of *S. exspinosus* were available at the time this study was made. Hence the comparison actually made does not have a bearing on the effect of the sex-intergrade character on rate of oxygen consumption[1] but is a study of rate of oxygen consumption of clones derived from a common mother, bred for a long time by parthenogenesis, and known to have become different in reproductive vigor, presumably through mutation.

The original Line 740 was taken from wild stock at Cold Spring Harbor in August, 1912. It has to date been reared for 698 parthenogenetic laboratory generations. Strain II originated from one of the substrains of this line in December, 1917, in its 136th partheno-

[1] As judged by routine laboratory observations of reproductive characteristics, there is no fundamental association between the sex-intergrade character and reproductive vigor except that when sex-intergradedness is very marked, reproductive capacity is reduced; and extremely intergrade individuals are generally entirely sterile. It is regretted that material for the study of oxygen consumption of moderately and highly intergraded strains was not available.

genetic generation. At the time this study was begun, it was in its 481st laboratory generation. Strains V and XVI, likewise substrains of Line 740, originated in December, 1917, and October, 1919, in the 135th and 205th generations, respectively. At the time this study was begun, they had descended, respectively, 420 and 437 parthenogenetic generations.

APPARATUS AND METHODS

The general cultural methods used in rearing these animals have been described elsewhere (Banta, 1921*a*, 1921*b*). The method of handling this stock preparatory to its use in determinations of oxygen consumption was to isolate in individual culture bottles a large brood of newly released young females from each strain. The animals were reared in the same culture medium and under as favorable and uniform conditions as could be provided. The determinations were made for these females on the day when they produced their first brood of young.

Simocephalus exspinosus (and all Cladocera) present considerable advantages for experimental work of this type. They are easily reared in the laboratory; their generation cycle is short; all the individuals of a clone are genetically alike; and they can be kept under close laboratory observation, thus affording a knowledge of their reproductive activity, general vigor, and precise state of development. These factors are of primary importance in experimental work of this type. We have been able to record our observations of oxygen consumption of individuals reared under a very close laboratory control.

Simocephalus exspinosus females, at the time of the release of their first clutch of young, have an average length of about 2 mm. Because of the small size of the individuals studied, it became necessary to employ an apparatus sufficiently sensitive to enable one to record minute amounts of consumed oxygen when the individuals were tested singly or in small groups. Thunberg's microrespirometer was employed as modified by Fenn (1927) to detect very minute quantities of oxygen. We have only slightly modified this apparatus to make it more suitable for work on Cladocera. To avoid the necessity of taking a large number of determinations, the apparatus was

made as sensitive as possible under the experimental conditions, by reducing its size.

The apparatus consisted of two bottles, each one about 8 cc. in capacity, connected to a horizontal capillary, graduated in millimeters and containing a minute drop of kerosene. The bore of the capillary in the apparatus employed in this work was about $\frac{1}{2}$ mm. A distance of 1 cm. in the capillary was equivalent to 0.0020573 cc. Since the reduction in oxygen pressure in the bottle containing the animals was equally shared by the two bottles, the oxygen consumed was actually equivalent to twice the volume of displacement of the kerosene drop. Between the horizontal capillary and each bottle, there was a three-way stopcock, making it possible either to connect the bottles directly to the capillary or to open them to the outside. In each bottle, attached to the stopper, was a small spoon. In one of these spoons were placed a few drops of the culture medium and also the animals whose rate of oxygen consumption was to be measured. In the other spoon was placed culture medium alone, equivalent in amount to the volume occupied by the animals and the culture medium in the first spoon. Each bottle contained $\frac{1}{2}$ cc. of 2 per cent sodium hydroxide for the absorption of carbon dioxide.

In general the method employed in making determinations was similar to that employed by Fenn (1927). After placing the animals in the chamber with both bottles and one end of the capillary opened to the outside, the apparatus was inserted in a Freas water bath with thermostat control, for one half hour at 25° C. The temperature was constant to 0.01°–0.02° C. During this period the apparatus came to equilibrium. The bottles were then connected only to the capillary. The rate of movement of the kerosene drop was recorded, and the oxygen consumption computed. Readings were taken each 15 minutes. The observations generally extended for a period of 1 hour after a uniform rate of oxygen consumption had been observed. This frequent making of observations served likewise as a check on the proper workings of the apparatus.

OBSERVATIONS

In Table I are shown the results obtained with Strain II, the normal line of *Simocephalus exspinosus*. Throughout this set of

experiments, four individuals were placed in the respiratory chamber. The individuals subjected to experimentation were isolated in individual culture bottles as soon as they were released from the brood chamber of the mother. The oxygen consumption was determined when the animals had released their first clutch of young. *Simocephalus exspinosus* normal females reach this stage in 6–7 days at summer laboratory temperature (about 22° C.). The average body length of the individuals tested is shown to be 2.23 mm. Numerical values are given showing the rate of oxygen consumption

TABLE I

OXYGEN CONSUMPTION OF *Simocephalus exspinosus* FEMALES STRAIN II, AT TIME OF SEXUAL MATURITY

No. of Individuals in Chamber	Age in Days	Average Length in Millimeters	O_2 consumption Cc. $\times 10^{-7}$	
			Per Groups Tested per Hour	Per Individual per Minute
4	6	2.38	31,271	130
4	6	2.39	31,682	132
4	7	2.14	32,917	137
4	7	2.22	38,266	159
4	7	2.30	33,328	139
4	7	2.20	28,391	118
4	7	2.30	34,974	146
4	6	2.04	25,099	105
4	7	2.08	30,860	129
Average		2.23		133

per hour for each group of four tested. The last column shows the oxygen consumption per individual per minute, this average being 133×10^{-7} cc.

In Table II are shown the experimental results obtained with Strain XVI, one of the strains which exhibited the sex-intergrade mutation. The number of individuals in the respiratory chamber was varied with the different sets of experiments. At times two, four, and five individuals were employed. The individuals in this group required as a rule between 8 and 9 days to reach sexual maturity, but in some cases this period was not different from the period required by normal females, namely 6 or 7 days. In this set of experiments the average body length of the individuals tested is shown

to be 2.04 mm. The average oxygen consumption per individual per minute was 76×10^{-7} cc. as compared with 133×10^{-7} cc. per individual per minute for Strain II.

TABLE II

OXYGEN CONSUMPTION OF *Simocephalus exspinosus* FEMALES
STRAIN XVI, AT TIME OF SEXUAL MATURITY

No. of Individuals in Chamber	Age in Days	Average Length in Millimeters	O_2 Consumption Cc. $\times 10^{-7}$	
			Per Groups Tested per Hour	Per Individuals per Minute
2	9	2.02	9,875	82
5	8	2.05	23,041	77
2	8	2.00	8,299	69
4	7	2.02	15,224	63
4	7	2.02	17,281	72
4	8	1.98	17,692	74
2	6	2.16	11,109	93
Average		2.04		76

Table III shows the results of a few experiments with still another intergrade strain, namely Strain V. Although the average body length of the individuals subjected to experimentation proved

TABLE III

OXYGEN CONSUMPTION OF *Simocephalus exspinosus* FEMALES
STRAIN V, AT TIME OF SEXUAL MATURITY

No. of Individuals in Chamber	Age in Days	Average Length in Millimeters	O_2 Consumption Cc. $\times 10^{-7}$	
			Per Groups Tested per Hour	Per Individuals per Minute
4	9	2.10	16,870	70
4	9	2.11	18,516	77
4	9	2.45	16,458	69
4	9	2.31	17,693	74
2	8	2.42	8,641	72
Average		2.28		72

to be slightly larger than that of the normal females tested, the average oxygen consumption per individual per minute is shown to be 72×10^{-7} cc., or as in the previous intergrade line tested, approximately one-half the rate of the individuals in Strain II.

DISCUSSION AND CONCLUSIONS

It is evident from the comparisons presented here of the rate of oxygen consumption of *Simocephalus exspinosus* that two of the clones subjected to experimentation showed a marked physiological difference from the other clone. Clones V and XVI showed a rate of oxygen consumption approximately one-half that of Clone II from which they were originally derived. The degree of difference in the physiological behavior of these two groups of animals is sufficiently large to preclude any doubt regarding the significance of the results obtained.

Previous history of the original normal stock, the circumstances under which new features appeared in the first intergrade individuals discovered, as well as the fact that these features were heritable in subsequent generations, manifestly indicate that they are mutations. The degrees of manifestation of the new morphological features were subject to further mutation and varied in their manifestation. They were very marked in many individuals; in other individuals sex-intergradedness was manifested very slightly; and in some not at all. The new clones possessed the potentiality for producing normal males, normal females, and all degrees of intergrades. For a long period during which the sex-intergrade characteristics were intensively studied, the two clones here studied did not differ appreciably from the normal clone in reproductive characteristics or general vigor. At the time this study was made, however, the intergrade strains subjected to experimentation were low in their manifestation of sex-intergradedness; and physiologically they were distinctly different from the clones from which they were derived, manifesting this diversity both in reduced reproductive capacity and differences in their rate of oxygen consumption.

In the study presented here we have an instance of a physiological mutation which can be tested experimentally. There are few records of such cases of mutations and evolutionary changes which have appeared under laboratory conditions, the workings of which can be tested by the use of quantitative physiological methods. Banta and Wood (1928*a*), working with *Daphnia longispina*, have reported a thermophilous mutation. From a mutant stock, reared for a long time by parthenogenesis, these two workers obtained by inbreeding

in sexual reproduction an individual whose thermal characteristics were very different from those of the parent clone. This individual and its descendants constitute a truly thermal clone which thrives only at relatively high temperatures. These same workers have a number of physiologically different Cladocera clones which have arisen during parthenogenesis or, as the result of genetic recombination, in sexual reproduction. It seems probable that such physiological mutations are relatively common among Cladocera and probably among other organisms as well. This would seem an excellent field for further study of oxygen consumption.

SUMMARY

Among Cladocera clones derived originally from a single clone and propagated entirely by parthenogenesis, morphological and physiological mutations occasionally arise.

Three such clones, one normal and two mutant clones, exhibiting relatively low vigor and low reproductive capacity, were used in a study of their rates of oxygen consumption by means of a respirometer modified from Fenn's modification of Thunberg's apparatus.

The normal clone was found to have an oxygen consumption of 133 times 10^{-7} cc. per individual per minute, while the reproductively weaker mutant clones had rates respectively of 76 and 72 times 10^{-7} cc. per minute. Hence the reproductively weaker clones consumed oxygen only slightly more than half as rapidly as the normal "sister"-clone.

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THE EFFECTS OF PURE SOLUTIONS OF SODIUM, POTASSIUM, AND CALCIUM CHLORIDES ON THE OXYGEN CONSUMPTION OF PLANARIA DOROTOCEPHALA

(Two figures)

OLGA THEODORE HESS
Zoölogical Laboratory, University of Chicago

INTRODUCTION

ATTEMPTS to cultivate planarian tissues *in vitro*, as well as regeneration work involving very small pieces of worms, have encountered difficulty in finding a suitable artificial, balanced salt solution which would give satisfactory results. In the course of extensive work carried out in this laboratory with *Planaria dorotocephala*, it was found that isolated cells of this species will live and pieces will undergo reconstitution in media of different osmotic pressures and that the fatal effect of certain media depended on the relative amounts of certain cations present in solution. Recently Murray (1928) found that a low proportion of potassium to calcium salt (about one-half by weight, as a maximum) is essential, while *P. dorotocephala* is indifferent to a considerable range of variation in the amounts of sodium and calcium chlorides, singly or together. Therefore, it was of interest to correlate the morphological changes under the influence of the variation in the salt content of the medium with the results upon their respiratory metabolism. With this idea in mind, a study has been made of the effect on oxygen consumption of the three chief cations contained in the physiological salt solution, e.g., sodium, potassium, and calcium. The results obtained are in agreement with Murray's conclusion with regard to the relative toxicity of these three cations.

Very little work has been done on the effect of the neutral salts on the respiratory processes, and particularly on oxygen consumption. Warburg (1908), studying the oxygen consumption of sea-urchin eggs, found that addition of NaCl to sea water gives a decided increase in the respiratory rate and that this increase is not proportional to the corresponding increase in osmotic pressure. He

also observed an increase in respiratory rate on addition of potassium, while addition of calcium chloride to sea water containing an excess of sodium brought the oxygen consumption back to its normal rate. Meyerhoff (1911) confirmed Warburg's results as to the accelerating effect of sodium and the depressing action of calcium chlorides. These findings are contrary to those of Loeb and Wasteneys (1911), who did not find much difference in the rate of oxidation of *Arbacia* eggs in sodium and potassium solutions as compared with mixtures of sodium and calcium chlorides. Brooks (1919) studied the effect of potassium, sodium, and calcium chlorides and their antagonism with regard to carbon-dioxide production of *Bacillus subtilis*. She observed an increase in carbon-dioxide production in lower concentrations and a decrease in higher concentrations, for all three cations—sodium, potassium, and calcium. The difference consisted in the concentration which gave the first depressing effect. Contrary to the findings of Lipman (1909, 1911), an antagonism was observed by Brooks (1919) between sodium and calcium chlorides. Simultaneously Gustafsen (1919) studied the effect of sodium and calcium chlorides and of their mixtures on the carbon-dioxide output in *Aspergillus niger*. His findings agree in general with those of Brooks, but the concentrations which produce a decrease in the respiratory rate are very high. The 2M concentration of sodium chloride showed an initial decrease in respiration followed by an increase amounting to about 10 per cent; the same phenomenon was observed in 1M concentration but was less pronounced. Calcium chloride also gave a rise in respiration in lower, and a depression in higher, concentrations. Gustafsen believes that the peculiar results obtained with higher concentrations of sodium chloride are due to two factors in the action of this salt. One is a chemical factor which tends to stimulate; while the other factor, the osmotic pressure, tends to depress the respiration. Inman (1921) found that hypertonic solutions of NaCl and of $CaCl_2$, as well as their mixtures in proportion 50:1, all cause decrease in the rate of carbon-dioxide production of wheat seedlings. Gray (1924) found that, on raising the K/Na ratio to 1/7, the oxygen consumption of the excised gill of *Mytilus edulis* is invariably increased. In the absence of the calcium ion the motor activity is practically abolished, while the oxygen con-

sumption does not show any reduction from its normal value. Therefore, Gray concludes that calcium does not enter into the reactions which are affected by the monovalent cations or into the oxidation of the products of motor activity.

Mayer and Plantefol (1926) found that pure solutions of sodium and potassium chlorides increase the respiration of mosses within the range of concentration 0.1M to 0.5M and decrease it in higher concentrations. Calcium-chloride solutions did not increase the rate of oxidation, but on the contrary decreased it in 0.1M and higher concentrations.

With regard to vertebrate tissue, Thunberg (1909) found that potassium and sodium chlorides do not alter the respiratory rate of the frog's muscle, while calcium affects the respiration of the tissue by lowering the oxygen intake and carbon dioxide output. Unger (1914) came to the conclusion that hypotonic solutions of sodium chloride have no effect on the oxygen consumption of the spinal cord of a frog, while sodium chloride accelerates the respiration in hypertonic concentrations. Hypotonic solutions of potassium chloride gave large fluctuations, and hypertonic concentrations showed decided decrease in oxygen consumption. This investigator did not succeed in obtaining any antagonistic effect of the monovalent and divalent cations with regard to the respiratory rate. Lange (1924) studied the effect of calcium chloride on respiration of the striated muscle of the frog. He found that concentrations between M/125 and 1M decrease the respiration rate, while M/625 solution of $CaCl_2$ gave an increase over 15 per cent. He explains these effects on the basis of the colloidal changes produced by the action of the calcium salt. Reinwein and Singer (1927) studied the oxygen consumption of mammalian liver and kidneys under the influence of change in osmotic pressure and the specific effect of the sodium, potassium, and calcium ions. They observed a rapid decrease in pure distilled water. They did not arrive at any definite conclusion with regard to the effect of neutral salts. In the absence of sodium bicarbonate, calcium and potassium chlorides depressed the respiratory rate; and a similar depression occurred in a physiological sodium-chloride solution. No definite results were obtained with experiments on the antagonism between potassium and calcium chlorides.

I desire to express my thanks to Professor C. M. Child, under whose direction the work was done, and to Dr. L. Hyman, Dr. R. S. Lillie, and Dr. R. W. Gerard for valuable suggestions and constant interest in the progress of the work. I wish also to thank the Departments of Physiology and Physiological Chemistry of the University of Chicago for the use of the constant-temperature room and other necessary equipment.

MATERIAL AND METHODS

As already mentioned in the introduction, *Planaria dorotocephala* was used during the entire work to be reported in this paper. This species of flatworm in this part of the country lives in spring water rich in calcium salts, which may explain some of the peculiarities of these worms with regard to a high tolerance of high concentrations of calcium in artificial media. Worms of 10–14 mm. length (i.e., medium size) were selected from the general laboratory stocks kept in well water which is also high in calcium. The salt concentration of this well water, based on the analysis made by the Illinois State Water Division, was given by Murray (1927). The worms kept in the laboratory were fed regularly three times a week on beef liver and were used for the experiments 4 days after feeding in order to avoid irregularities due to the increased respiration which, according to previous investigations (Hyman 1919*b*), requires 4 days after the food intake to come to its basal level.

Two methods were used in determining the oxygen consumption of *P. dorotocephala:* the titration method of Winkler and the micro-respirometer method. The latter proved to be more satisfactory for this type of work, particularly with long exposures of worms to various salt solutions.

The procedure in determinations by the Winkler method was described by Hyman (1919*a*) and was extensively used in this laboratory in the work with flatworms and other small aquatic forms. Therefore, this method as used by the author will be described only briefly. Fifty worms of uniform size (10–14 mm. in length) were put in Erlenmeyer flasks of approximately 250 cc. capacity. Freshly boiled and filtered well water, which was allowed to cool down to 20° C., was then carefully siphoned into the flask; and the latter was

stoppered air-tight and kept in a water bath at 20° C±0.5° C. for 1 hour, after which period the flasks were reversed several times to give uniform mixture of the contents, and a sample was siphoned into a small bottle of approximately 60 cc. capacity, and the oxygen was determined by titration with m/200 sodium thiosulphate against iodine. From a comparison with the samples of well water taken at the beginning of the experiment and kept for 1 hour at the same temperature as the experimental flasks, the amount of oxygen consumed by the worms during 1 hour was estimated in cubic centimeters per hour. The determination of the normal respiratory rate was carried out in this manner during two successive hours. The worms were then subjected to pure solutions of sodium, potassium, and calcium chlorides, or of their mixtures; and the determination was carried on for two or three successive hours in the same manner as in well water. The same worms were used for a series of experiments with concentrations of salts which gave no change in the respiratory rate or gave only small fluctuation from the normal, while in high concentrations which gave an immediate change in oxygen consumption, particularly with potassium-chloride solutions every time a new lot of worms was used.

The crystalline salts of NaCl, KCl, and $CaCl_2$ $2H_2O$ used throughout the work were those of Baker's Chemical Company. The solutions were made up freshly every time by adding the necessary amount of salt to tin distilled water which was previously boiled in order to avoid the effect of acidity due to the presence of carbon dioxide. The solutions were then raised to a pH of 8.0 by adding sodium bicarbonate in a concentration which required 3 drops per 500 cc. of the salt solution to bring it to the desired pH. The pH was determined with the use of two indicators, phenol red and thymol blue, by comparison with standard sets.

The microrespirometers used in the second method were the ordinary Barcroft differential manometers (Barcroft, 1914) with caproic acid as filling fluid. The respiration chambers were of the shape of small Erlenmeyer flasks, with a flat and wide bottom, and a capacity of about 35 cc. The experiments were carried out in a constant-temperature room at 19.5° C. The flasks were immersed in a water bath of the same temperature; the water in the water bath

was kept in motion by means of a stirrer. The manometers were connected with a shaker, so that, together with the respiration chambers, they were shaken during the entire experiment at a uniform rate which gave the maximum diffusion. The differences observed in the temperature of the room and that of the bath varied within $\pm 0.2^{\circ}$ C. The manometers were calibrated by the physical method described by Hoffman (1913). The carbon dioxide was absorbed in the usual manner by m/2 NaOH, and the amount of oxygen consumed was calculated in millimeters per hour.

During the preliminary experiments with normal respiration in well water it was found desirable to introduce the worms into the respiration chambers at least several hours before the beginning of the oxygen-consumption determination in order to avoid the fluctuations brought about by the restless condition of the worms. Best results were invariably obtained when the worms were left overnight in the respiration chambers. Twenty worms of 10–14 mm. length were used for each experiment; and the volume of fluid was 10 cc., which were added from a calibrated pipette.

The worms were weighed before they were introduced into the chambers. The method of weighing is described by Hyman (1919*b*). Special care was taken in order to obtain uniform drying on the filter paper. The worms were never allowed to lose their shiny appearance, because, if they do so, then they may be easily injured in removing them from the filter paper. With some practice the error due to the difference in water elimination from the surfaces of the worms can be brought down to 3 per cent. In the experiments involving long exposures (over several days) of worms to solutions of various neutral salts, worms were usually weighed at the end of the oxygen-consumption determination in order to avoid the stimulation of weighing before the experimental period.

The normal respiration rate was first determined in boiled and filtered well water; and then, after careful rinsing, avoiding as much as possible unnecessary stimulation of worms, the salt solution to be tested was added. The solutions were made up from stock solutions of KCl, NaCl, and $CaCl_2\ 2H_2O$ of molar concentrations and were made up in double-distilled (copper-tin) water of pH 7.0. The solutions were raised to pH 8.0 with sodium bicarbonate. The pH was

determined with the use of the same indicators as in the first method. Readings were made every half-hour in the case of short-time experiments and every hour when experiments lasted 1 day or longer. The error in reading the manometer amounts to 0.002 cc. From 40 to 60 minutes were allowed for the equilibrium to be established before the first reading was made.

There are several advantages in the use of the manometric determination over the titration method. First, the method is much more sensitive. Second, the pH during the experiment remains constant, owing to the absorption of carbon dioxide. And third, the specific action of the salt used in pure solution is more pronounced, owing to the fact that with the smaller number of worms and the smaller glass surface of the respiration chambers, as compared to the 250 cc. Erlenmeyer flasks used in the Winkler method, less salt from well water remains behind on the mucus-covered wall of the containers and therefore there is less chance for antagonistic action.

In order to estimate the relative toxicity of the three salts used in the experiments on oxygen consumption, it was necessary to determine the susceptibility of *P. dorotocephala* and its behavior in these salt solutions. For this purpose ten worms of uniform size (*ca.* 14 mm.) were introduced into each of a series of small Erlenmeyer flasks and rinsed three times with the solution to be tested. After this careful rinsing, 50 cc. of the solution were added and the observations made at regular intervals. The solutions were made up in the manner described above for the microrespirometer determinations of oxygen consumption, and the pH was raised to 8.0 with sodium bicarbonate. No attempt was made to buffer the solutions, because any buffer employed would introduce, if not a toxic, at least an antagonistic factor. Therefore, the pH was maintained at an approximately constant level by adding fresh medium every hour, except for the worms which were left overnight, for example in the case of calcium chloride.

THE EFFECT OF DISTILLED WATER

In the course of experimentation with *Planaria dorotocephala* it was observed that this species of flatworms is more susceptible to distilled water than several others, for example *P. maculata*. At

first this great toxicity of the distilled water was thought to be due to the fact that the water used was metal distilled and not glass distilled. The work of Murray has shown that there is no difference in resistance of *Planaria dorotocephala* to metal- or glass-distilled water; therefore the lethal effect of distilled water can be attributed only to the low osmotic pressure and absence of salts necessary for maintenance of life.

Either single- or double-distilled water constituted the solvent of all the salt solutions employed during the entire work reported in this paper. This water, as already mentioned, was obtained for oxygen-consumption work from a tin still with the first method (Winkler) and from a copper-tin still in manometric determinations. The same copper-tin double-distilled water was used during the susceptibility experiments; its pH varied between 7.0 and 7.4.

Duration of life and behavior of worms in distilled water.—The duration of life of *Planaria dorotocephala* in distilled water showed great variation, and this was also observed during experiments on oxygen consumption with both methods. After a long period of experimentation it was established that this variation was entirely due to the difference in treatment of worms during the transfer from their natural environment (i.e., well water to distilled water) and also to the difference in the entire procedure in the case of the Winkler method as used by Hyman and the microrespirometer determination as carried out by the author.

If ten worms of medium size are placed in a thoroughly cleaned glass flask and rinsed three times with distilled water raised to pH 8.0 by addition of minute quantity of sodium bicarbonate and then exposed to 50 cc. of this water renewed every hour, the duration of life is approximately 6 hours; while it is approximately 30 hours when the water is added without rinsing and remains unchanged during the entire period of exposure.

In order to establish definitely the effects of the difference in procedure in the exposure of worms to distilled water, three sets of susceptibility experiments were carried out by the author, including each four lots of worms (ten worms of medium size, in 50 cc. of distilled water, the volume of liquid and the number of worms being the same as used in all the susceptibility experiments with the salt solu-

tions). These four groups of worms were treated each in a different manner; and the results which were essentially similar in all the three sets of experiments were averaged and are presented in the form of a graph (Fig. 1), plotted on the basis of observations of five stages of disintegration according to the method used in this laboratory for comparing differences in susceptibility.[1]

The differences in treatment of worms before and during the exposure to distilled water were as follows: Procedure A: the worms were rinsed three times during the transfer from well water to distilled water, and the medium was freshly renewed every hour. Procedure B: the worms were not rinsed, but well water was thoroughly decanted and 50 cc. of distilled water added and freshly changed every hour. Procedure C: the worms were rinsed three times during the transfer from well water to distilled water, but the distilled water was not changed during the entire period of exposure. Procedure D: the worms were not rinsed, but well water was thoroughly decanted and 50 cc. of distilled water was added and not changed during the entire period of exposure. In this manner the effect of rinsing, as well as the effect of the periodic renewal of the medium, could be evaluated.

The first reaction of the worms to distilled water is very similar to that in other more or less irritating media. It consists of increased muscular activity, twisting, and head movements. The signs of irritation last approximately half an hour or less, after which period

[1] The method serves merely to show graphically differences in susceptibility of the first zooid in different animals and under different conditions (Child, 1915). For purposes of record, five stages are distinguished: (I) intact animals; (II) from the beginning to the completion of head disintegration; (III) disintegration of the anterior half of the postcephalic region; (IV) disintegration of the posterior half of the postcephalic region; and (V) complete disintegration of the body wall of the first zooid. Each stage is given a numerical value (e.g., 40, 30, 20, 10, 0) for the stages I to V, respectively. In experiment the number of animals in each stage is recorded at intervals; and these numbers, multiplied by the numerical value of each stage and divided by the number of animals in the experimental lot, give a numerical expression of what may be regarded as the average condition of the lot at the time of record. These numbers, plotted as ordinates against times of observation in hours from the beginning of the experiment, give a curve of susceptibility such as the curves in Figure 1. The posterior zooids are not included in such records, since they differ in different individuals. In all cases, however, except where acclimation is concerned, the posterior zooids are completely disintegrated before the disintegration reaches the posterior end of the first zooid.

the animals become quiet and, if not disturbed, remain attached to one place with retracted heads and the entire animal greatly shortened. After a period of quiescence and slight swelling, which may last several hours, death gradually takes place; its first visible indication is the disintegration of heads.

Signs of disintegration were first observed with the procedure A and appeared approximately 4 hours after exposure to distilled water. These were soon followed by disintegration in lots B and C,

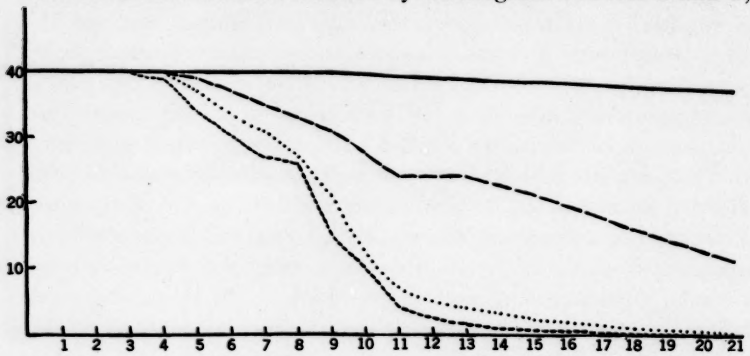

FIG. 1.—Curves showing differences in susceptibility of *Planaria dorotocephala* due to variations in treatment during exposure to distilled water. Procedure A (short dashes); Procedure B (dotted line); Procedure C (long dashes); Procedure D (continuous line). Abscissas represent time in hours from beginning of experiment, and ordinates the numerical values at each period of observation (see footnote, p. 17).

although the latter showed a much slower advance of disintegration than the first lot. The lot D was the last to die; a slight disintegration, usually of a single worm, began around the tenth hour of exposure; and a large percentage of worms was in normal condition at 30 hours after exposure, while all the first three lots were usually completely disintegrated much earlier.

The disintegration in distilled water shows the general gradient effect in the first zooid. Beginning with the head, disintegration of the body wall progresses posteriorly in the anterior zooid, the margins disintegrating before the median region. That distilled water has no direct paralyzing effect on the muscular apparatus of the worms is seen from the fact that the worms never lose their adhesion until the disintegration is advanced.

The data presented in Figure 1 indicate that there is a great difference in the survival time of animals between procedures A and D. On the other hand, the difference in results obtained from procedure B and C shows that the most important factor in the variation of the method of procedure is the renewal of the medium rather than the rinsing. The rinsing may have an initial stimulating effect, as will be shown by the results of experiments on oxygen consumption, but it does not shorten life as much as the repeated renewal of the medium. The only possible explanation of the effect on survival time of the different procedures seems to be that the animals give off salts into the water and so decrease its toxicity. Unless the volume of water is very large in relation to number of animals, without change of water these are sufficient in amount to increase survival time greatly, or, with a sufficiently large number of animals in sufficiently small volume of water, to permit life to continue indefinitely. With frequent change of water, they are rapidly exhausted and death occurs early. This idea finds support in the results obtained by Adolph (1925) for the earthworm and the frog: the earthworm, when immersed in distilled water, was found to give off salts, although in very small amounts; and the same was found for the frog in hypotonic media. Procedure B can be compared with that in the Winkler method for oxygen determination, where the worms were rinsed only slightly and never more than once.

Procedure C is essentially that employed in the manometric determination of oxygen consumption except that there a smaller volume of liquid in relation to the number of worms was used, and carbon dioxide was absorbed, giving a constant pH, which of course was absent in the susceptibility experiments, where the water was not renewed. Under these conditions the duration of life is greater than in the susceptibility experiments, evidently because of the smaller volume of fluid, since the pH is not sufficient to bring about earlier death.

Oxygen consumption in distilled water, as measured by the Winkler and the microrespirometer methods.—Six preliminary 2-hour experiments on oxygen consumption of *Planaria dorotocephala* in tin distilled water, which was previously boiled and raised to pH 8.0 by addition of sodium bicarbonate, were carried out. At the time when

well water was replaced by distilled water, the flasks containing the worms were only slightly rinsed, never more than once. These preliminary experiments showed that there is no change in the respiratory rate on transfer to distilled water, and the rate remains essentially the same for two successive hours, fluctuating always within the limits of experimental error. Furthermore, a series of thirty-three 4-hour experiments and six 3-hour experiments were performed as distilled-water controls during the first part of the work with various salt solutions by the Winkler method. These experiments show also fluctuations in the respiratory rate which do not permit any positive conclusion. Out of thirty-nine experiments, four have shown a depression of respiration on the third hour of exposure, and nine out of thirty-three experiments gave a decrease in the rate of respiration on the fourth hour of exposure. The decrease noticed during the third hour of exposure to distilled water mounted to 16–25 per cent of the normal rate, and at the end of the fourth hour of exposure the decrease observed varied between 11 and 38 per cent of the normal. There is no indication of even a slight increase in the respiratory rate within the entire series with distilled water covering the period of 4 hours of exposure measured by the Winkler method.

Seven experiments with the microrespirometer method, which include three short experiments of 6 and 7 hours of exposure and four long-time experiments covering 30 hours of exposure, and which thus include the period of beginning disintegration, show the same variation, with a tendency toward depression within the period concerned during the experiments with the first method. The depressions noticed in the manometric determinations of oxygen consumption were not of constant occurrence and were small, varying between 4 and 12 per cent of the normal respiratory rate.

Not the slightest increase in oxygen consumption was ever observed during the first 7 hours of exposure to distilled water with the second method. Therefore, the respiration of *P. dorotocephala* can be considered normal, with a slight tendency toward depression during the first few hours of exposure.

Table I gives the data of four long-time experiments which were continued beyond the period of beginning disintegration in order to find out whether the respiration in distilled water changes during

disintegration of the animals. These four experiments show clearly that with the beginning disintegration of animals, which in case of distilled water corresponds with the beginning death-changes, the oxygen consumption rises.

THE EFFECT OF SODIUM CHLORIDE

Duration of life and behavior of worms in pure sodium-chloride solutions.—Solutions of pure sodium chloride in concentrations from 0.002M to 0.1M were tested. The muscular activity consisting of rhythmic waves of contraction is extremely vigorous in the three

TABLE I

FOUR EXPERIMENTS ON OXYGEN CONSUMPTION OF *Planari dorotocephala* IN DISTILLED WATER (MICRORESPIROMETER METHOD)

RESPIRATION PERIODS IN HOURS AFTER EXPOSURE	PERCENTAGE OF INCREASE IN OXYGEN CONSUMPTION			
	Experiment I	Experiment II	Experiment III	Experiment IV
23–24	0	0	0	0
24–25	7	10	0	0
25–26	0	37	.....	12
26–27	.....	.....	13	20
27–28	25	47	22	28
28–29	57	100	.....	.....
29–30	50	62	.....	.....

higher concentrations (0.1M, 0.04M, and 0.02M), but no co-ordinated locomotion takes place. With decrease in concentration the intensity and duration of this rhythmic activity decrease, until in 0.01M sodium chloride it is practically absent. In 0.1M solution the contraction very soon goes over into a permanent tetanus, so that addition of fresh medium does not call forth a new wave of contraction as in the case of lower concentrations. During complete paralysis the animals lie on their sides, curved so that their ends almost touch each other, with their ventral surfaces concave. No disintegration of the animals in 0.1M solution takes place for many hours after their death in the solution. This is apparently due to the high osmotic pressure of the solution, which extracts water in large amounts from the bodies of the worms. In order to determine the time of death of the worms in the 0.1M solution, it was necessary to take them out at different periods after exposure and return them

to well water. The parts affected disintegrate within an hour. The disintegration always begins at the anterior end and shows a definite gradient along the axis. In this manner the death-point of planarians in the 0.1M solution was estimated to lie between 10 and 11 hours after exposure. In animals returned to water after 9 hours of exposure, the heads disintegrated and postcephalic regions recovered very slowly. After an exposure of 10–11 hours no recovery was possible, and complete disintegration took place.

The worms always begin to lose their ability to adhere to glass with the beginning of paralysis. The latter takes place in 2 hours after exposure to 0.04M solution, in 5 hours in 0.02M, and in 9 hours in 0.01M solutions of sodium chloride. In 0.01M solution the disintegration follows soon after complete paralysis; while in the higher concentrations the disintegrative process is delayed so that, while in the three lower concentrations (0.01M, 0.004M, and 0.002M) the worms were entirely disintegrated within 18 hours, in 0.04M and 0.02M solutions the disintegration had only begun. In the two lowest concentrations (0.004M and 0.002M) of sodium chloride no definite paralysis or loss of adhesion was observed, and the disintegration began about the ninth hour of exposure and proceeded rapidly to completion. Instead of tetanus and shrinkage, a certain amount of swelling could be observed in these lowest concentrations.

During the entire work a considerable amount of fluctuation in the degree of susceptibility to the pure solutions of various salts was noticed, depending on the condition of the stock culture from which the worms were selected, the time which elapsed after the last feeding, and the treatment of the worms in transferring them to pure salt solutions. This last point was discussed with regard to distilled water. With toxic concentrations of sodium chloride the rinsing has a greater effect than in distilled water, because the degree of initial muscular activity, and consequently the increase in respiratory metabolism, depends on the amount of rinsing at the beginning of the experiment.

Oxygen consumption as determined by the Winkler method.—Over forty short experiments with the lower concentrations of sodium chloride, beginning with 0.001M, were performed. No definite change in the respiratory rate was observed during the entire series

of experiments, the fluctuations being always within the limits of the experimental error. With increasing concentration, as shown in Table II, the first accelerating effect was obtained with 0.066M sodium chloride. This concentration and also 0.1M bring about very strong contraction, which is followed soon by a complete paralysis. During the first hour of exposure the respiratory rate rises 30 to 40 per cent, but it returns to its normal level after the second hour of exposure.

The same amount of sodium chloride as in 0.1M solution, when added to boiled and filtered well water, produces no increase in the

TABLE II

RESULTS OF A TYPICAL EXPERIMENT, SHORT-TIME, WITH SODIUM CHLORIDE AT EACH OF THE HIGHER CONCENTRATIONS USED (WINKLER METHOD)

(Oxygen consumption given as cubic centimeters of oxygen consumed per hour)

	CONCENTRATION OF NaCl				
	0.1	0.066	0.04	0.028	0.02
First hour	0.12	0.19	0.14	0.13	0.14
Second hour	0.09	0.15	0.12	0.15	0.13
Well-water control	0.09	0.15	0.12	0.15	0.13

respiratory rate. The contraction observed in the pure solutions of this concentration was also abolished.

Oxygen consumption as measured with the microrespirometer.—Twenty-two experiments with six different concentrations of sodium chloride were carried out. None of the experiments was of less than 5 hours of exposure; during the course of experimentation it was found that the respiratory rate in sodium-chloride solutions remains practically constant in the three lower concentrations for over 6 hours; therefore it was desirable to continue the experiments up to the time of disintegration. Several experiments were performed covering a period of 10 and 11 hours of exposure.

The animals were rinsed three times with the solution to be tested before the beginning of the oxygen consumption determination; this was done in order to avoid, as far as possible, the effect of the salts which may remain on the surfaces of the worms and in the respiratory chamber. In considering the period of the initial ac-

celeration in high concentrations of sodium chloride, a certain degree of stimulation which is due to repeated rinsing of worms should be taken into account.

TABLE III

TYPICAL EXPERIMENTS ON OXYGEN CONSUMPTION IN SODIUM-CHLORIDE SOLUTIONS AT EACH OF THE CONCENTRATIONS USED COVERING THE PERIOD OF 6 HOURS IN THE EXPERIMENTAL SOLUTIONS (MICRORESPIROMETER METHOD)

(Oxygen consumption given as cubic millimeters of oxygen consumed, calculated per hour)

RESPIRATION PERIODS IN HOURS AFTER EXPOSURE	CONCENTRATIONS IN MOLS.					
	0.1	0.04	0.02	0.01	0.004	0.002
1.0–1.5	51	54	58	.....	.....	.....
1.5–2.0	44	40	49	38	36	35
2 –3	44	43	46	36	42	39
3 –4	40	.....	46	36	42	42
4 –5	39	47	47	33	42	42
5 –6	39	43	.....	30	.....	42
Respiration in well water*	40	34	44	35	42	41

* Averages of three successive manometer readings.

Table III gives actual experimental values; these are later included in the averages presented in Table IV.

TABLE IV

AVERAGE PERCENTAGE INCREASE IN OXYGEN CONSUMPTION WITH SODIUM CHLORIDE AT EACH OF THE CONCENTRATIONS USED (MICRORESPIROMETER METHOD)

RESPIRATION PERIODS IN HOURS AFTER EXPOSURE	CONCENTRATION IN MOLS.					
	0.1	0.04	0.02	0.01	0.004	0.002
1.0–1.5	41	34	36	.....	.....	.....
1.5–2.0	29	14	5	4	−3	−5
2 –3	20	16	9	0	0	0
4 –5	7	15	7	0	0	0
Number of experiments	3	4	2	3	4	3

From the susceptibility experiments we know that the exposure to 0.1M, 0.04M and 0.02M solutions of sodium chloride is accompanied by vigorous muscular contraction if the worms are rinsed several times before the transfer to the experimental solution is made. An increased oxygen consumption would naturally be ex-

pected during the first, and possibly the second, hour of exposure, depending on the duration and strength of the contraction waves.

As already pointed out in the discussion of the methods, the microrespirometer determination of oxygen consumption gives no information concerning the rate of respiration during the first hour of exposure, but the readings taken during the first half of the second hour still show a large increase in oxygen consumption, which evidently is a result of the muscular stimulation under the influence of sodium-chloride solution. By the end of the second hour of exposure a drop in the amount of oxygen consumed is noticed in the three higher concentrations used, although it has not yet reached its normal level at this time.

The susceptibility experiments have shown that during the second half of the second hour of exposure the contraction usually ceases and the animals are either completely paralyzed, apparently in more or less contracted condition (in 0.1M), or at least have become quiet (0.04M and 0.02M). It is possible that the contraction lasts longer in the 0.04M and 0.02M solutions in the oxygen consumption experiments, as compared with the susceptibility experiments, because of the continued shaking of the manometers and of the respiration chambers through the entire experiment. The shaking can have no effect on the duration of muscular activity in the 0.1M solution where complete paralysis takes place immediately after the cessation of the muscular activity. In 0.04M and 0.02M solutions, on the other hand, the period of muscular stimulation may be prolonged by the shaking; this possibility is suggested by the fact that during the susceptibility experiments new waves of contraction were easily brought about by the renewal of the experimental medium before the onset of complete paralysis, i.e., 2 hours for 0.04M and 5 hours for 0.02M solutions of sodium chloride.

The respiratory rate in all of the three higher concentrations used was found to return gradually toward its normal level, which it may or may not reach, depending apparently on the degree of the initial muscular stimulation. During the third or fourth hour of exposure the oxygen consumption usually reaches a certain level at which it remains for several hours. This level is apparently higher the greater the percentage of increase of oxygen consumption for a given con-

centration at the time of exposure of the worms to pure salt solutions. In 0.01M solution of sodium chloride after the very slight contractile activity has ceased and the animals merely creep actively before they become quiet, the oxygen consumption rises only very slightly above its normal level, as shown in Table III. After this very slight increase, the respiratory rate returns to its normal level, which it maintains all through the 6-hour experiment. Results with 0.004M and 0.002M solutions, as given in Table III, indicate an initial depressing effect, but the decrease was not of constant occurrence and the rate always returned to normal on the third hour of exposure.

TABLE V

PERCENTAGE OF INCREASE IN OXYGEN CONSUMPTION IN FIVE LONG-TIME EXPERIMENTS WITH SODIUM CHLORIDE AT VARIOUS CONCENTRATIONS (MICRORESPIROMETER METHOD)

RESPIRATION PERIODS IN HOURS AFTER EXPOSURE	CONCENTRATION IN MOLS.				
	0.1	0.01		0.004	
1.0– 1.5	27	.....	.....	.....	.....
1.5– 2.0	10	8	0	0	0
2 – 3	10	0	0	0	0
3 – 4	0	0	0	0	0
4 – 5	0	0	0	0	0
5 – 6	0	0	.....	.....	0
6 – 7	.....	14	21	7	0
8 – 9	17	14	.....	39	18
9 –10	30	18	27	67	57
10 –11	.....	21	40	72	80

Table IV summarizes the results of eighteen experiments covering a period of 5 hours of exposure to sodium-chloride solutions, and shows essentially the same character of response as the single experiments in Table III.

In order to determine whether there is a change in respiration at the time of death of the worms in the sodium-chloride solutions, five long-time experiments were carried out for a period of 11 hours, and the results are presented in Table V.

The data for the first 5 hours of the experiment with 0.1M and the first one with 0.01 solutions were used in figuring out the averages for that period of exposure.

All the long-time experiments show that the oxygen consumption

begins to rise slowly at the end of the seventh hour of exposure, i.e., just before the beginning paralytic effect in 0.01M solution and the death of the animals, which apparently takes place in the majority of cases about the ninth hour of exposure.

In higher concentrations of sodium chloride there are two definite rises in the respiratory rate: one at the beginning of the experiment and the other at the end.

The initial increase in oxygen consumption which is observed only in higher concentrations is apparently due to the increased metabolism as a result of intense muscular activity. In lower concentrations which do not stimulate the muscles to contract the initial increase in oxygen consumption is entirely absent.

The increase in oxygen consumption which takes place in all the concentrations of sodium chloride used and which is pronounced in the entire series of experiments about the eighth or ninth hour of exposure (i.e., the time coinciding with the approach of death in pure sodium-chloride solution) is evidently connected with the death-changes which take place in the protoplasm, and is of no direct interest to this investigation.

On the basis of the entire series of experiments with both methods we may conclude that there is no direct effect of sodium chloride in the concentrations used on the oxidative processes of *Planaria dorotocephala*.

The absence in the experiments with the Winkler method, except for the 0.1M and 0.066M solutions of the initial increase in respiration observed with the microrespirometer, is probably due, at least in part, to differences in procedure in the two methods. In the Winkler experiments the animals were only slightly rinsed, never more than once, and then rather superficially, in order to avoid stimulation; furthermore, the animals were used during several days—sometimes for a whole week—without being removed from the flasks, and therefore the latter were coated with a large amount of mucus which undoubtedly contained salts. Moreover, the Winkler method at best is much less delicate than the respirometer method. In the 0.1M solution, after a large initial increase, the respiration drops down to its normal level with the use of both methods, but less rapidly with the microrespirometer method.

THE EFFECT OF POTASSIUM CHLORIDE

The duration of life and behavior of worms in pure potassium-chloride solution.—Pure solution of potassium chloride in concentrations from 0.04M to 0.002M were used. Higher concentrations have proved to be instantaneously lethal. The effect of potassium chloride differs greatly from that of sodium chloride in that disintegration is not delayed and follows rapidly the state of complete paralysis. In all concentrations disintegration was noticed to occur not later than 2 hours after the worms were completely paralyzed.

Very slight contraction waves of an entirely different character than those observed in sodium-chloride solution were noticed only in the highest concentrations used (e.g., 0.04M), while in 0.002M there was only a certain amount of irritation expressed in rapid crawling movements. The muscular contraction observed in 0.04M solution was not rhythmic and lasted only for a few minutes, after which period a gradual relaxation of the entire muscular apparatus set in. The progress of the paralytic effect in potassium chloride is very rapid and terminates in complete paralysis without recovery on return to well water. The approximate times at which no recovery takes place on return to well water are as follows:

0.04M, 1½ hours after exposure
0.02M, 3 hours after exposure
0.01M, 5 hours after exposure
0.004M, 7 hours after exposure
0.002M, 9 hours after exposure

These periods also correspond to the time at which complete paralysis takes place and the pharynges are protruded; they are not at all absolute, and vary with the physiological state of the animals, e.g., their size and the time which has elasped after the last feeding. The shorter the period between the time of feeding and the exposure to potassium-chloride solution, the greater is the toxicity of the pure salt solution. The lethal effect of potassium chloride is much more pronounced than that of sodium chloride.

The region of the pharynx is the first to be attacked by potassium chloride, but this is evidently due to the peculiar phenomenon which accompanies paralysis of the worms in potassium-chloride solutions, the protrusion of the pharynges, either out of the mouth or through

the dorsal wall, producing a wound. In both cases the pharynges after a while separate from the body of the worms and are found lying free in the solution. This complete detachment of the pharynges is characteristic for potassium chloride and was never observed in other salts, in spite of the fact that in the highest concentrations of calcium chloride the protrusion of pharynges through the mouth usually took place. The injury connected with the protrusion of the pharynx led to early disintegration of the middle body region, so that the animals often separated into two pieces which continued to disintegrate. At the same time, the head disintegrates and the disintegration of the anterior body region progresses posteriorly, the lateral margins preceding. The protrusion of the pharynges occurs in many anesthetics and in various other agents, but the conditions which bring it about have not yet been determined. The detachment of the pharynges may be explained as a result of the swelling of the tissues and cytolysis. An absorption of water by the cells was noticed in approximately 0.05M potassium chloride by Murray (1928), who also noticed that the muscle cells were the first to cytolyze. The disintegration in potassium-chloride solution, once started, proceeds very rapidly, particularly in higher concentrations, which is contrary to the effect of sodium-chloride solutions and indicates absence of coagulative changes in the protoplasm under the influence of this salt.

Oxygen consumption as measured by the Winkler method.—Twenty-six experiments with potassium chloride at various concentrations were carried out. The average percentages of increase in oxygen consumption obtained from twenty-one experiments are given in Table VI. The other five experiments are single experiments with concentrations higher than or between those presented in the table and are not sufficient in number to permit any definite conclusion.

Table VI shows clearly that potassium chloride in the highest concentration increases oxygen consumption during the first 2 hours of exposure. The average percentage of increase in 0.01M solution lies within the experimental error; only one experiment out of five showed an increase of oxygen consumption of 23 per cent at the end of the first hour of exposure, while the other four experiments showed

slight fluctuations from normal, all within the limits of experimental error. In 0.002M solution, which also shows an increase within the limits of experimental error, three out of five experiments showed normal rate of respiration. All concentrations except 0.02M show a definite increase in oxygen consumption for all respiration periods longer than 1 hour. In the highest concentration, 0.02M, the increase apparently falls off slightly during the experiment, but the change is not great and may be due to experimental error. In general the respiration continues to rise during the progressing paralysis, as is indicated by the respiration in 0.02M and 0.004M solutions at the

TABLE VI

AVERAGE PERCENTAGE OF INCREASE OF OXYGEN CONSUMPTION IN POTASSIUM-CHLORIDE SOLUTIONS AT EACH OF THE CONCENTRATIONS USED (WINKLER METHOD)

(Numerals in parentheses represent the number of experiments used in averaging the percentage increase in oxygen consumption)

RESPIRATION PERIODS IN HOURS AFTER EXPOSURE	CONCENTRATIONS IN MOLS.				
	0.02	0.013	0.01	0.004	0.002
First	25 (5)	23 (2)	4 (5)	0 (2)	5 (5)
Second	21 (3)	30 (2)	12 (3)	0 (2)	10 (5)
Sixth	.....	.....	75 (2)	58 (2)	.....
Seventh	18 (2)	30 (2)	37 (2)	.....	.....
Eleventh	.....	.....	.....	.....	20 (3)

end of the sixth hour of exposure and in 0.002M at the end of 11 hours of exposure. The rise apparently continues up to the beginning of disintegration; when the latter sets in, the respiratory rate, as measured by the Winkler method, decreases or remains constant. The drop may be determined by the presence of carbon dioxide produced by the animals or the increase in acidity resulting from death of some of the cells, which in the absence of a buffer in the solution makes the medium sufficiently acid and thereby depresses the rate of oxidation.

Oxygen consumption as measured by the microrespirometer method.—Eleven experiments with the three higher concentrations of potassium chloride (0.04M, 0.02M, and 0.01M) show a definite increase in the oxygen consumption and substantiate the results obtained with the Winkler method. The experiments were not carried beyond the beginning of disintegration. The results are presented in Table VII.

Eight experiments with 0.004M potassium chloride showed a certain amount of variation. During the first half of the second hour of exposure five out of seven readings showed a depression in the rate of respiration varying between 6 per cent and 16 per cent of decrease from the normal. The other two readings were normal respiratory rates; no increase was observed in the entire series 0.04M solution of potassium chloride during the first 2 hours of exposure. This corresponds exactly to the results obtained with the Winkler method.

TABLE VII

AVERAGE PERCENTAGE OF INCREASE IN OXYGEN CONSUMPTION IN POTASSIUM-CHLORIDE SOLUTIONS AT EACH OF THE CONCENTRATIONS USED (MICRORESPIROMETER METHOD)

(The numerals in parentheses indicate the number of readings used in averaging)

RESPIRATION PERIODS IN HOURS AFTER EXPOSURE	CONCENTRATIONS IN MOLS.			
	0.04	0.02	0.01	0.004
1.0–1.5	20 (3)	.....	.....	− 7 (7)
1.5–2.0	31 (3)	31 (2)	11 (4)	0 (5)
2.0–2.5	50 (5)	23 (2)	16 (4)	7 (7)
2.5–3.0	.....	45 (2)	32 (4)	4 (6)
3.0–3.5	.....	50 (2)	29 (4)	13 (2)
3.5–4.0	.....	77 (3)	42 (5)	8 (5)
4.0–5.0	.....	.....	.....	7 (2)
5.0–5.5	.....	.....	.....	17 (3)
5.5–6.0	.....	.....	.....	17 (3)
6.0–7.0	.....	.....	.....	35 (2)
8.5–9.0	.....	.....	.....	51 (2)
9.0–9.5	.....	.....	.....	51 (2)

The amount of depression observed cannot be considered significant from a quantitative point of view because the average lies within the limits of the experimental variation. It is evident from Table VII that the same applies to all of the data on 0.004M up to and including the fifth hour of exposure. Beginning with $5\frac{1}{2}$ hours of exposure, a definite increase in the respiratory rate is observed in all the experiments continued to this period and beyond. As indicated by the susceptibility experiments, this is approxmately the time of beginning paralysis; animals are completely paralyzed about 7 hours after exposure, after which time no recovery is possible on return to well water. Thus the decided rise in respiration in potassium-chloride solution seems to accompany the progressing paralytic changes. The same is true for the higher concentrations of potassium chloride.

The entire series of experiments on the effect of potassium chloride on oxygen consumption as measured by both methods shows definitely that the rate of respiration is greatly affected by this salt and that the increase in respiration due to the influence of the potassium salt in the medium corresponds to and follows the progress of the paralytic effect of the potassium ion.

THE EFFECT OF CALCIUM CHLORIDE

Duration of life and behavior of worms in pure calcium-chloride solutions.—The susceptibility experiments, as well as the respiration experiments with the manometric method, have revealed an interesting fact, namely, that *Planaria dorotocephala* can live for very long periods of time, perhaps indefinitely, in pure solutions of calcium chloride of concentrations ranging from 0.02M to 0.002M. Lower concentrations than 0.002M were not used by the author, so that the lower limit of that range is not definitely established. In higher concentrations, as, for example, in 0.04M, the worms live for 48 hours; while in 0.1M solution they die and begin to disintegrate after 6 hours of exposure.

The animals were treated in the usual manner before exposing them to the salt solutions; i.e., they were rinsed three times, and 50 cc. of the experimental salt solution were added and periodically renewed. In 0.1M solution, in which the worms lived only a few hours, the medium was changed several times during this short period; in concentrations in which the worms lived over a month (after which period no more observations were made) and remained in perfectly normal condition, the medium was usually changed only twice in 24 hours, at morning and evening. In order not to introduce any other salts together with organic substances into the medium, the worms were not fed during the period of experimentation either in susceptibility experiments or in the respiration experiments; consequently they underwent a considerable decrease in size and became less resistant to handling, i.e., were easily injured during weighing. In all of the solutions of the above-mentioned range in which the worms survived a period of 1 month or more active regeneration took place in cases of fission. Observations on the comparative rate of regeneration and on head frequency as compared to normal re-

generation in well water were not attempted, and remain as a possibility for future studies.

In connection with the respiration experiments it was desirable to determine whether the loss of weight by the worms in the pure calcium-chloride solutions proceeded at the same rate as in ordinary water or whether the calcium ion in the complete absence of other ions influenced in some way the rate of decrease in weight. This question arose in connection with the idea that the increase in respiration which is observed in calcium-chloride solutions subsequent to a decided depressing effect might be associated with a more rapid starvation in the experimental solutions as compared with well water.

In the attempt to answer this question two additional series, each consisting of twenty worms in each of the concentrations mentioned above, were weighed at intervals through a period beginning with the fourth and ending with the eighteenth day after the last feeding, i.e., a period before starvation increases the respiratory rate of the worms (Hyman, 1919*b*). The average percentage decreases of body weight are presented in Figure 2.

The early loss of body weight is greatest in the highest concentration used (0.04M), and the animals die within 48 hours after a rapid decrease in weight. During the first 2 days of exposure the loss of body weight in 0.004M, 0.01M, and 0.02M does not differ appreciably; at 5 days the difference between 0.02M and 0.004M is obvious, but less between 0.02M and 0.01M. In both series of worms in 0.02M fissions occurred and disintegration was observed in some of the posterior fission pieces about the sixth day. The earlier loss of body weight in 0.002M solution is least as compared with the other concentrations of calcium chloride, but the curve as shown in Figure 2 included only 7 days because disintegration began on the sixth day in one of the lots. The lot which survived showed practically no difference in decrease of weight as compared with the lots in 0.004M calcium chloride. The loss of weight always followed, as we see, the increase in osmotic pressure of the solution. Adolph (1925), in his work on the loss of body weight in flatworms, considers the decrease in weight as due primarily to loss in water content under the influence of change in osmotic pressure of the medium; this is possibly an explanation of the initial loss of body weight or for a compara-

tively short period of exposure to the experimental medium; the greater decrease in weight subsequent to the possible reduction in water content must be explained differently. It is known for *Planaria dorotocephala*, as well as for other flatworms, that excess of calcium in the medium brings about an overproduction of mucus and that the production of the latter is reduced in the absence of this cation. Increased production of mucus in pure calcium-chloride me-

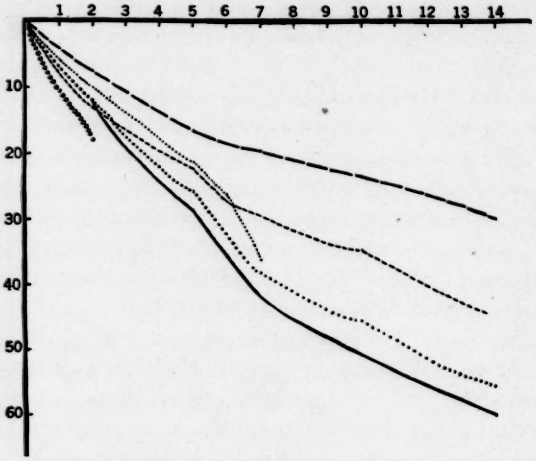

FIG. 2.—Curves showing percentage of decrease in body weight in well water and in various concentrations of pure calcium chloride. Well water (long dashes); 0.002M (small dots); 0.004M (short dashes); 0.01M (large dots); 0.02M (unbroken line); 0.04M (circles). Abscissas represent time in days, and ordinates percentage decreases in body weight.

dium may be responsible for the decrease in weight of the worms in these experiments as compared to the normal loss of weight in well water.

Intense muscular activity, consisting of bending, twisting, elongation, and lifting of the body, was observed in 0.1M and 0.04M solutions of calcium chloride. The muscular activity lasted only about 15 minutes in the 0.1M solution and was followed by a complete paralysis in the elongated condition and loss of adhesive power. Another interesting characteristic of the reaction of the worms to pure calcium-chloride medium in high concentrations (0.1M and

0.04M) is a clumping of the worms during the period of intense muscular activity; the worms gather in one spot of the container and stick to each other with their surfaces, which appear as though covered by an opaque silvery film in case of the highest concentration of salt used; in 0.04M this clumping phenomenon is only a temporary one. For a number of hours the animals in 0.1M solution remain intact, in a completely paralyzed condition with pharynges protruding through the mouth. They gradually become very thin and flat, which undoubtedly is due to a reduction in the water content of the body; this dehydration effect is much more obvious if one compares such a lot of worms with those in potassium chloride, where the reverse phenomenon, i.e., imbibition of water, was observed. Just before the disintegration of the worms in 0.1M solution of calcium chloride, extremely large amounts of slime are given off, so that the latter are clumped by strands of stiffened opaque mucus. A typical feature of disintegration in high concentrations of calcium chloride is the complete disintegration of the margins beginning with the head region, while the ventral and dorsal surfaces are yet intact; the high susceptibility of the lateral margins can be explained by the hyperfunction of the gland cells, which produce abnormal quantities of mucus and, because of their activity, become highly susceptible. After the disintegration of the marginal areas a slight agitation of the medium in the container may induce an immediate falling apart of the entire organism.

If the worms are returned to well water after 5 hours of exposure and before the disintegration of the margins has occurred, they do not recover, but very soon disintegrate; the disintegration in well water after exposure of worms to the higher concentrations of calcium chloride shows the usual gradient, beginning always with the head region; after periods of many hours, when the disintegration of the entire zooid has long been complete, the posterior zooid region may remain without undergoing disintegration and may even recover; the same phenomenon was also observed with 0.1M solution of sodium chloride. This indicates either a change in the solution or a very great ability of the posterior zooid to acclimate.

Oxygen consumption as measured by the Winkler method.— Twenty-six short-time experiments with calcium-chloride solutions

of 0.066M and 0.002M concentrations were carried out, covering a period of 2 hours of exposure to experimental solutions. Depression occurred only in 0.04M, and here also mainly on the second hour of exposure, i.e., with the second addition of experimental solution. The average decrease in oxygen consumption during the first hour amounts to 5.8 per cent, which has no significance because it lies within the limits of experimental variation with this method. The average decrease in respiration in six experiments during the second hour of exposure amounts to 31 per cent, two showing normal rate of respiration. The absence of a definite decrease in oxygen consumption in 0.066M and during the first hour in 0.04M may be due to the fact that the depressive effect of calcium chloride is compensated by the muscular stimulation which occurs in high concentration of the salt. Only one out of eight experiments with 0.02M calcium chloride showed a decrease in respiration, while the remaining ten experiments with 0.0133M, 0.01M, 0.004M, and 0.002M gave a normal rate of respiration during both hours of exposure.

Oxygen consumption as measured by the microrespirometer method.—The results obtained with the microrespirometer method substantiate the results obtained by the Winkler method. Twenty lots of worms were tested for oxygen consumption in pure solutions of calcium chloride in concentrations ranging from 0.1M to 0.002M. As pointed out in the section on survival in pure calcium-chloride solutions, this species adjusts itself to pure calcium-chloride medium of a range from 0.02M to 0.002M. It is possible that 0.002M is not the lower limit of survival in calcium chloride, but no lower concentration than 0.002M was tested by the author.

Table VIII gives the data for the effect of higher concentrations of calcium chloride on oxygen consumption. The results with lower concentrations not included in the table, i.e., 0.004M and 0.002M, show fluctuations in the respiratory rate within the limits of the normal experimental variation, which for the entire series with calcium chloride (twenty experiments) are +5.8 per cent and −5.6 per cent.

The absence of decrease in oxygen consumption in 0.1M during the first 2 hours of exposure may be due to the compensation of the depressing effect of calcium chloride on the oxidation process by the

stimulation of the musculature by the calcium ion in very high concentrations, producing abnormally strong muscular contortions. The death-changes in 0.1M solution begin early, which accounts for the decided rise in oxygen consumption already on the third hour after exposure and beginning disintegration during the sixth hour. The depressing effect is pronounced in 0.04M and is comparatively slight, but not entirely absent in 0.02M solution; while 0.01M shows no effect on the respiratory rate, neither immediately nor within $5\frac{1}{2}$ hours after exposure.

TABLE VIII

AVERAGE PERCENTAGE OF CHANGE IN OXYGEN CONSUMPTION IN CALCIUM-CHLORIDE SOLUTIONS AT THE HIGHER CONCENTRATIONS USED (MICRORESPIROMETER METHOD)

(Numbers represent average percentage of change in oxygen consumption)

RESPIRATION PERIODS IN HOURS AFTER EXPOSURE	CONCENTRATION OF CALCIUM CHLORIDE IN MOLS.			
	0.1	0.04	0.02	0.01
1.5–2.0	4	−25	− 6	0
2.0–2.5	.....	−18	−10	0
2.5–3.0	14	.....	4	4.5
5.0–5.5	23.6	0	0	4
Number of experiments	3	2	3	4

Because of the high tolerance of *Planaria dorotocephala* to calcium chloride, it was of interest to determine whether the respiratory rate remains unaffected during the first 2 weeks of exposure or whether it differs from the respiration of the control worms in well water. For this purpose, eleven of the above-mentioned lots of worms which were tested immediately after exposure to various concentrations of calcium chloride were continued for approximately 2 weeks in the pure calcium-chloride medium, which was renewed every day; and the oxygen consumption was determined at various periods. These eleven experiments do not include any in 0.1M solution because the worms die in this concentration within 6 hours after exposure; only one lot of worms survived during 48 hours of exposure in 0.04M solution, the other set was lost on the second day by injury during weighing. Table IX summarizes the results for the long-time experiments. The figures in each of the five subdivisions of each vertical

column of Table IX represent percentages of change in oxygen consumption obtained from successive readings on the same experimental lot at intervals of $\frac{1}{2}$ or 1 hour after the beginning of the period indicated in the first column of the table.

TABLE IX

PERCENTAGE OF CHANGE IN OXYGEN CONSUMPTION, LONG-TIME EXPERIMENTS IN CALCIUM CHLORIDE AT VARIOUS CONCENTRATIONS USED (MICRORESPIROMETER METHOD)

CONCENTRATION IN MOLS.	0.04	0.02	0.02	0.02	0.01	0.004	0.004	0.004	0.002	0.002	0.002	WELL WATER I	WELL WATER II
First day (on transfer from well water)	−23		−13			− 6			−10			0	−4
	−42	− 1	− 7	−10	− 2	− 3	− 1	1	− 4	8	3	4	8
	−28	−19	− 7	− 4	3	0	− 1	− 9	− 5	−1	−1	−4	1
		3	− 2	11	0	0			− 8	2	−6	0	−4
	11	− 4	− 1	5	0	0					−3		3
After 24 hours	−22	−11				−13	6	27	9	0			
	−11	2					7	2	2	0			
	− 5	10				7	12		−13	−2			
	− 2	4				− 6	13		3	0			
							1		−15	0			
							4		−10	7			
After 48 hours	41												
	53												
	16												
	50												
After 96 hours		7				12						−3	4
		11	−32	−13		9						−2	0
			−12			3						7	−9
		12	4	5		12						1	1
		13	8			19							
			−11			1							
After 288 hours		− 5	17		5		19	41		−9	0	2	1
		2	26		16		12	19			−2	−2	0
		15	30		28		8	36		−7	−2	−3	
		1	12		16			39		2	−8		
					19			9		−4			
					22								

In 0.04M and 0.02M solutions of calcium chloride there is a decrease in respiration immediately on exposure, but about the third hour the respiration begins to return to its normal level. Exactly the same initial depression followed by a return to normal repeats itself in 0.04M as well as in 0.02M solutions after the solution is renewed

24 hours later. Disintegration begins in 0.04M solution around or shortly after 48 hours of exposure, and with the death-changes the oxygen consumption of the worms in calcium chloride rises as in sodium-chloride experiments, and continues to do so during the process of disintegration. The figures for the 96-hour periods show much variation for which no interpretation can be offered. After 12 days (288 hours) the increases in 0.02M and 0.01M become more pronounced, while the results for 0.004M vary, and with the available data do not permit any definite conclusions; both lots of worms tested show an initial rise followed by a considerable fall in the respiratory rate toward normal; moreover, oxygen consumption in one of these lots which showed an increase in respiration on the twelfth-day period showed normal respiratory rate when tested on the twentieth day after exposure to the experimental solution. There is no doubt that the respiration rate remains essentially normal in 0.002M solution of calcium chloride.

The entire series of experiments on oxygen consumption with pure calcium-chloride solutions measured by both methods shows a decided depressing effect of this salt on the respiration of *P. dorotocephala* if the concentration exceeds 0.01M. In all of the concentrations used above 0.01M, the decrease in respiration was followed sooner or later by a gradual approach to normal. Renewal of the solution was usually followed by a new decrease and another approach to normal. In higher concentrations (0.02M and 0.01M) there is an indication of an earlier increase in the rate of oxygen consumption as compared with well-water controls, but additional experiments are necessary for a definite conclusion. A 0.002M solution of pure calcium chloride was found not to affect the respiratory rate of *P. dorotocephala*.

OSMOTIC PRESSURE AND SPECIFIC EFFECT OF THE THREE CATIONS

In order to draw a comparison between the specific action of sodium, potassium, and calcium chlorides on *Planaria dorotocephala*, it is necessary to consider the relative osmotic pressure of the solutions which are used during the experiments. The fresh-water organisms, as contrasted with the marine forms, have only a limited capac-

ity to adjust themselves to changes in osmotic pressure of their surrounding medium. From the foregoing experiments on susceptibility as well as on oxygen consumption, it is possible to estimate, at least approximately, the range of osmotic pressures which intact individuals may withstand. The concentrations used during the entire work reported in this paper, when corrected for ionization, cover the range from 0.0039 osmolar (corresponds to freezing-point depression of 0.002M NaCl or KCl; likewise boiled and filtered well water) to 0.2597 osmolar (i.e., 0.1M $CaCl_2$). Thus all the experimental solutions except distilled water and the lowest concentrations of sodium and potassium chlorides have a higher osmotic pressure than the boiled well water, which was used for normal controls during the entire work reported in this paper. The unboiled well water contains a larger amount of salts than the boiled and filtered, and gives, therefore, a greater freezing-point depression (approximately, Δ 0.02°), which changes with the season and the amount of rain. This freezing-point depression corresponds approximately to that of 0.004M calcium chloride. Although the solutions used in this work are in the majority of cases hypertonic to the boiled and filtered well water, they are not necessarily hypertonic to the animals, because the flat worms, like most fresh-water forms, maintain a higher osmotic pressure than their surrounding medium. The internal osmotic pressure of *P. dorotocephala* is still unknown because of lack of adequate methods of determination, and consequently the point of isotonicity is obscure; all we know at present is the range of osmotic pressures within the limits of which the animals can survive during a certain period of time, or indefinitely. This particular work is another attempt to establish the limits within which acclimation to various salt solutions is possible from the point of view of respiratory changes.

If we consider the results on oxygen consumption from the point of view of osmotic pressure changes in the medium, we find that the lower limit employed in the experiments is that represented by the boiled well water, while the upper limit is 0.02M calcium chloride, which, when corrected for ionization, gives 0.05502 osmolar concentration. This species is apparently capable of withstanding change from one to the other of these extremes without much dif-

ficulty; but whether this entire range in osmotic pressures is withstood is not completely determined for calcium chloride. At present we know that the animals can live for over a month and perhaps indefinitely in pure calcium chloride of a concentration between 0.05592 osmolar, the upper limit of the osmotic range, and approximately 0.005 osmolar (0.002M calcium chloride). The range between the osmotic pressure of the latter concentration and the osmotic pressure of the boiled and filtered well water remains for future study.

If we now turn our attention to the differences in results for oxygen consumption in the three salts under consideration during the period of 6 hours after exposure, then we find that the upper limit of the osmotic range for potassium chloride is very low, somewhat higher for sodium chloride, and highest for calcium chloride. This gradation in reactions of the worms to these three cations corresponds to the order of the amounts in which potassium, sodium, and calcium are present in the natural environment of the worms, the ratio being approximately 1:3:49, respectively (according to the analysis of the well water made by the Illinois State Water Survey Department). The actual ratio, of course, varies considerably in different waters in which the animals are able to live. The natural spring water in which the worms are found has, like the well water, a high calcium content.

DISCUSSION

The experiments reported in this paper seem to show a great difference in the specific action of sodium, potassium, and calcium chlorides in solutions of approximately the same osmolar concentrations. Potassium chloride was found to increase the respiratory rate of *Planaria dorotocephala*, and the increase follows the progress of paralytic effect. Sodium chloride has no direct effect on respiration except through the stimulating action upon the musculature of the animals. This increase in oxygen consumption due to muscular activity of the animals was only a temporary phenomenon, and the respiration returned toward its normal level in all the concentrations of sodium chloride in which the muscular activity was not immediately followed by the onset of death-changes. Pure 0.002M calcium-

chloride solution, which corresponds osmotically to approximately 0.005M sodium chloride, gives a normal rate of respiration during several days of exposure. Higher concentrations of calcium chloride give an initial decrease of respiratory rate with a gradual return toward normal. Within the same range of osmotic pressure the effects of sodium and potassium chlorides are distinctly and characteristically different.

The present conception of the action of salts is that their primary effect is to change the normal permeability of the plasma membrane, this change being followed by a secondary effect on the internal protoplasm. According to Osterhout (1912, 1915), in the case of plant cells the permeability is increased by sodium and potassium salts and decreased by calcium salts. R. S. Lillie (1909, 1910) reached the same conclusion as regards permeability changes in *Arenicola* larva and the sea-urchin egg (in the latter case, sodium being a more powerful agent than potassium). Spek (1921) studied the penetration of sodium, potassium, and calcium chlorides into the rhizopod, *Actinosphaerium eichhorni*. The cell proved to be most permeable to potassium, less to sodium, and still less to calcium ions. In their experiments on *Ameoba*, Chambers and Reznikoff (1926) have found that the effects of sodium, potassium, and calcium differ with regard to the plasmalemma and the internal protoplasm of the cell and that the monovalent cations (Na and K) are decidedly more toxic than bivalent (Ca and Mg). They interpret their results on the internal protoplasm as a liquefying action of sodium and potassium and a solidifying action of calcium and magnesium chlorides. This interpretation of the results of Chambers and Reznikoff was criticized by Heilbrunn (1928), who reinterpreted their results as indicating that sodium and potassium chlorides always produce an increase in viscosity of the protoplasm which would coincide with the results which he himself obtained with *Arbacia* eggs, while the viscosity is reduced on exposure of the eggs to pure calcium chloride or to excess of calcium chloride in a balanced isotonic solution. He agrees with the possibility of the coagulative action of calcium chloride when directly injected into *Amoeba*, as is the case in Chambers' and Reznikoff's experiments, and considers this phenomenon as a secondary change occurring under the influence of a calcium salt. Heilbrunn

bases his conception of the action of these salts on the assumption of a positively charged protoplasmic colloid.

These various changes in the physicochemical state of the protoplasm under the influence of sodium, potassium, and calcium chlorides may take place in *Planaria dorotocephala*, but we know practically nothing about them; the striking difference in the effect of sodium and potassium chlorides on the process of respiration and the difference in the progress of disintegration in, for example, 0.04M solutions of each of these two salts suggests a difference in penetration of these two cations, as well as in their action on the internal tissues of the worms. As in the case of *Amoeba*, potassium is more toxic than sodium chloride. A partial interpretation of this difference can be obtained from the study of the penetration of these two cations through a collodion membrane. In a free solution the mobility of the potassium ion as compared to the sodium ion does not differ appreciably (K = 64.67; Na = 43.55), while the difference, according to Michaelis (1925), is exaggerated within the membrane. "The differences are so large that the K ions may be considered as almost impermeable in comparison with the H ion; on the other hand the Na ion and still more the Li ion is almost impermeable in comparison to the K ion." The supposed cause of the retardation in mobility is the increased friction of the water envelope in the capillary canals of the membrane.

Another interesting point which was already emphasized in the preceding chapters is that of increase in length of life in the pure salt solutions as well as in distilled water when not renewed. This suggests a certain modification of the environment by the animal which permits life to continue longer. Such modification probably consists in the giving-off of salts by the animals.

This paper represents only the first steps in the study of the effects of the chief constituents of balanced salt solutions on the survival and oxygen consumption of *Planaria dorotocephala*. Some data on antagonism between potassium and calcium chlorides with regard to respiration and on the effect of change in the balance of the salts of Ringer solution have already been obtained by the author and will be published in the near future.

SUMMARY

1. Changes in oxygen consumption of *Planaria dorotocephala* were determined in distilled water, and in pure solutions of sodium, potassium, and calcium chlorides immediately on transfer from well water and during the entire time of survival of the worms in these experimental solutions. The range of concentrations of the salts used was 0.1M to 0.002M for sodium and calcium chlorides and 0.04M to 0.002M for potassium chloride. The solutions were raised to pH 8.0 by addition of a small quantity of sodium bicarbonate.

2. The determinations were carried out at 20° C by two methods: the Winkler method and the use of the differential Barcroft manometer.

3. The respiration of *P. dorotocephala* in distilled water may be considered normal with a slight tendency toward depression during the first few hours of exposure. The respiration rate begins to rise about the twenty-sixth hour of exposure, and this increase in oxygen consumption is due to beginning death-changes.

4. The duration of life in distilled water raised to pH 8.0 by addition of sodium bicarbonate varies greatly with the variation in treatment of the worms during the transfer from well water to the experimental solution, and depends largely on the frequency of renewal of the medium during the course of the experiment.

5. Sodium chloride in the concentrations used has no direct effect on oxygen consumption. An initial increase in respiratory rate was observed during the period of intense muscular activity on exposure to 0.1M, 0.04M, and 0.02M solutions of sodium chloride, but the increase was only temporary and the respiratory rate approached normal level after the cessation of the muscular activity. Another rise in respiration occurred in all of the concentrations used with the beginning death-changes, and persisted during the period of disintegration.

6. Potassium chloride was found to be more toxic than sodium chloride. The duration of life in equimolar concentrations of potassium chloride as compared with sodium chloride is shorter. The disintegration in potassium chloride follows soon after complete paralysis of the worms and proceeds very rapidly. Protrusion of pharynges, followed by their complete detachment, was observed in potassium-chloride solutions.

7. The oxygen consumption in 0.04M, 0.02M, and 0.01M potassium chloride rises immediately on exposure to the solutions and continues to rise during the period of paralysis and the following disintegration. In 0.004M solution the respiration at first remains normal, fluctuating within the limits of experimental variation, and begins to rise about the fifth hour of exposure.

8. *Planaria dorotocephala* can live over a month and perhaps indefinitely in pure calcium-chloride solutions of a range between 0.02M and 0.002M. The lower limit of this range is not determined because no solutions of lower concentration than 0.002M were used by the author. Concentrations of 0.04M and 0.02M gave an initial decrease in respiration followed by a return to normal. This decrease was repeated with every renewal of the medium. The animals live only 48 hours in 0.04M calcium chloride; and an increase in the respiratory rate is observed with the beginning death-changes, shortly before the disintegration begins.

9. A difference in the rate of body-weight loss was observed in calcium-chloride solutions as compared to the loss of weight in well water.

10. The upper limit of the osmotic range for the three salts used decreases in the order: $CaCl_2$. NaCl and KCl, which corresponds to the ratio in which these cations are present in natural waters: 49:3:1.

11. The great tolerance of *P. dorotocephala* to large amounts of calcium chloride is explained on the basis of the high proportion of this salt present in the natural environment of the worms.

12. An explanation of the difference in the action of sodium and potassium on the basis of the difference in their ionic mobility in a membrane as determined by Michaelis for a collodion membrane is suggested.

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CONTROL OF SEX IN CLADOCERA. VI. BY TREATMENT WITH DRUGS[1]

ARTHUR M. BANTA
Carnegie Institution of Washington, Cold Spring Harbor, New York

AND

L. A. BROWN
Zoölogical Laboratory, State University of Iowa, and
Carnegie Institution of Washington

FEMALES of *Moina macrocopa* release their first brood of young at a somewhat later time when crowded together in a bottle of culture medium than when reared singly. This retardation in development was shown to be closely associated with male production (Banta and Brown, 1929*d*). It was also shown (Banta and Brown, 1929*e*) that mothers experimentally treated in such a manner as to overcome part of the retardation caused by crowding, correspondingly produce fewer males than crowded sisters not so treated. The present paper presents data from experiments devised to test the effect of a reduced rate of development of the mothers, induced by drugs, on the sex of their young.

EXPERIMENTAL

The general procedure in these experiments was practically the same as in others previously reported. Single broods of young females were obtained from the regular stock of this species, which had been reared parthenogenetically for several hundred generations in the laboratory, and divided among three (or two) bottles of culture water. One of these bottles was kept as an untreated control (given an equal amount of distilled water), while drugs which might reduce the rate of development were introduced into the other bottles. Such treatment was given during the adolescent (third) instar and before or during the critical period for sex determination. This period had previously been located at about 4 hours, at 20° C., before the end of this instar (Banta and Brown, 1929*c*). The effect of

[1] From the Department of Genetics, Carnegie Institution of Washington, Cold Spring Harbor, New York.

these drugs on the rate of development was found by obtaining the time of release of the first broods of young by the control and test mothers. Following the release of the young, their sex was determined. The number of mothers per bottle ranges from five to eight in most cases, constituting what we call "semicrowded" bottles in distinction to bottles having ten or more mothers, which are known as "crowded" and which produce on the average at least 40 per cent males. The semicrowded mothers usually produce from 10 to 25 per cent male young. This method of handling the mothers insures a more delicate test of the male-influencing character of the treatment used than if the control gave a much higher male production.

Treatments with many different anesthetics and depressing agents were tried; and four, namely, chloretone, potassium cyanide, phenyl urethane, and ethyl urethane, which showed promising results, were rather extensively used. It is generally assumed that anesthetics inhibit to a large extent the general metabolic processes of an organism. This was stated by Child (1910) and reaffirmed by Allee (1912). Potassium cyanide in weak solutions has been used to test the metabolic levels of animals or parts of animals (Child, 1913) or to reduce the metabolic rate of animals (Allee, 1912). The effect of both chloretone and KCN is likened to that produced by decreasing the oxygen supply. The urethanes belong in the group of hypnotics (Sollman, 1917) and should produce somewhat similar effects as chloretone, opiates, and other such drugs. It should be pointed out that the interest was not centered on the specific action of the drug, but only on the production of a measurable, differential rate of development between the control animals and their treated sisters together with effects upon male production. The results obtained justified the choice of drugs.

Chloretone.—In all, about one hundred and seventy-five individual experiments, involving over four thousand mothers, were tried with chloretone. Not all of these experiments were completed, since in some cases many of the test animals died, produced too few young, or were excessively retarded and thus subject to extraneous environmental influences not affecting the controls. Table I presents data for one hundred and six complete experiments. It will be seen that in general the females from a single brood were divided equally

among three bottles. The treatment with chloretone was graded in the preliminary experiments until a concentration was reached which would delay the release of the animals from 30 minutes to 2 hours. The chloretone solution used was a specified amount of a saturated solution at 20° C., which gave a concentration in the culture bottle of from 0.04 to 0.08 per cent. The exact amount present in our cul-

TABLE I

EXPERIMENTS WITH CHLORETONE

(Release records of the young are mostly in terms of hours after that of the respective controls. Time of treatment is given as the third instar or in number of hours before egg laying.)

No. of Experiments	Control		Chloretone (Light Treatment)				Chloretone (Heavier Treatment)			
	Total Young Sexed	Percentage of ♂'s	Total Young Sexed	Percentage of ♂'s	Release Record	Time of Treatment	Total Young Sexed	Percentage of ♂'s	Release Record	Time of Treatment
7	377	33.7	307	47.2	0.74 hr. later	± 12 hrs.				
6	556	63.7	570	69.8	later	third instar	554	66.6	later	third instar
6	395	11.9	388	22.6	no record	third instar	330	5.2	no record	third instar
6	704	5.5	683	14.8	later	late third instar	579	18.5	later	late third instar
7	289	23.9	188	43.1	no record	no record	161	37.9	no record	no record
5	316	35.1	324	71.3	2.5 hrs. ± later	no record	338	56.2	6 hrs. ± later	no record
10	686	47.4					692	38.4	later	late third instar
2	352	13.1					533	28.0	2.6 hrs. later	middle third instar
10	2,067	3.7	2,039	1.2	1.75 hrs. later	7–2 hrs.	978	11.4	2.02 hrs. later	7–2.5 hrs.
10	762	0.5	811	3.1	0.25 hr. later	14–6 hrs.	280	0.5	0.28 hr. later	14–6 hrs.
7	882	5.8	999	8.4	0.46 hr. later	5–4 hrs.				
12	743	47.4	655	74.4	1.55 hrs. later	12–5 hrs.	613	68.7	2.0 hrs. later	12–5 hrs.
8	526	12.4	1,039	14.4	0.75 hr. later	9–4 hrs.				
10	752	14.8	859	35.5	0.50 hr. earlier	8–6 hrs.	726	45.7	1.0 hr. earlier	8–6 hrs.
Totals and Averages										
106	9,407	18.9	8,862	23.9	0.76 hr. later		5,784	35.0	1.11 hrs. later	

ture bottles cannot be accurately determined as most of the treatments consisted of a few drops of this solution in the regular culture water or in pond water at each half-hour or hour interval over a period of from 2 to 8 hours before the critical period, in order that some concentration of the drug would be effective at this crucial time in the development of the egg. We had sufficient evidence to indicate that chloretone, as such, does not long persist in our culture medium. The treatments can, however, be divided into those that were relatively light and those that were heavier, and the data are tabulated in the table in terms of such a division.

It will be seen from the table that of those series of experiments for which release records are available, only two[1] of the nineteen cases of chloretone-treated mothers failed to show a retardation (as compared with their respective controls) in release of their young. The average delay in release for the lighter treatment is about 45 minutes and for the heavier treatment 1 hour and 5 minutes. In addition to this delay in the release of the young, the treated mothers produced more males than the untreated controls in nineteen out of twenty-three individual cases (in one case there was no difference); while the three differences in the reverse direction are relatively slight. The average male production of the controls for all experiments was 18.9 per cent males; that for the lighter treatment, 23.91 per cent males; and for the heavier treatment, 35.01 per cent males. In other words, the male percentage of the heavier treatment shows an increase of about 85 per cent over that given by the controls; and this increase in male production is closely associated with a marked retardation of the mothers' development, as indicated by the release of their young.

Phenyl urethane.—Table II presents the accumulated data of eighty-one experiments in which phenyl urethane was the drug used. The general results, while of the same order as that found for chloretone, are somewhat more consistent. Nine series of experiments were tried. In one series the release record was not obtained; in two cases there was no appreciable difference between the time of release of the control and treated mothers (and no significant difference in male production); but in six series of experiments the animals treated with phenyl urethane released their young later than the control mothers. In all of these cases, except one, the retarded mothers produced a higher percentage of males than the control mothers. In the single exceptional series, the control and treated mothers produced practically the same proportion of male young, but the treated animals released much later and gave a greater difference than in any of the other series of experiments. This is probably due, as is known definitely in other cases, to the fact that the treatment was not administered

[1] These two cases so clearly run counter to our other experience with chloretone treatments that we feel certain some supplementary effect must have operated here *after* the critical period.

in time to influence the sex of the forthcoming young (i.e., was applied after the critical period) but did have a pronounced effect on the rate of development of the mothers following this time. This aberrant release record is not included in the average release record given in the last line of the table. For the entire table, including some non-significant series, the controls show 23.35 per cent males and the tests, 38.9 per cent males. For the last four series in which the treat-

TABLE II

EXPERIMENTS WITH PHENYL URETHANE

No. of Experiments	Control		Treated with Phenyl Urethane		
	Total Young Sexed	Percentage of ♂'s	Total Young Sexed	Percentage of ♂'s	Release Record
12	1,274	7.2	1,275	13.9	all later
3	118	0.0	181	18.2	no record
10	1,544	29.3	1,413	29.9	no difference
10	557	38.4	531	40.8	no difference
10	901	9.1	708	8.5	3.1 hrs. later
6	138	45.6	105	75.2	1.4 hrs. later
8	585	13.8	425	39.5	0.4 hr. later
12	1,014	15.2	978	64.8	0.7 hr. later
10	887	56.5	1,943	59.2	0.1 hr. later
Totals and Averages					
81	7,018	23.4	7,559	38.9	
Totals and Averages for Last Four Series					
36	2,624	30.5	3,451	58.8	0.64 hr. later

ments were satisfactory and for which release records were obtained, the average time of release was 38 minutes later than that for the controls, and the male percentage was 58.84 compared with 30.45 for the controls—nearly twice as large a male percentage as that for the controls.

Ethyl urethane.—A few experiments (Table III) in which ethyl urethane was used are given for the sake of completeness. In two series of experiments no significant difference is found in the proportion of males produced by the treated and control mothers, and no record of the release was obtained; in two cases the treated mothers

produced many more males than the control mothers; and in one of these latter cases this increased male production is associated with a delay of 1.8 hours in the time of release of the treated mothers.

TABLE III

EXPERIMENTS WITH ETHYL URETHANE

No. of Experiments	Control		Treated with Ethyl Urethane		
	Total Young Sexed	Percentage of ♂'s	Total Young Sexed	Percentage of ♂'s	Release Record
5	353	36.5	263	35.3	no record
6	409	12.2	400	10.0	no record
5	493	0.0	346	18.8	no record
10	1,156	3.9	863	14.6	1.8 hrs. later
Totals and Averages					
26	2,411	9.3	1,872	17.3	

TABLE IV

EXPERIMENTS WITH POTASSIUM CYANIDE

No. of Experiments	Control		Treated with Potassium Cyanide		
	Total Young Sexed	Percentage of ♂'s	Total Young Sexed	Percentage of ♂'s	Release Record
2	213	11.3	190	26.3	no record
10	843	42.0	975	49.4	no record
12	1,303	14.0	1,117	8.9	0.33 hr. later
16	1,728	15.6	3,332	18.7	0.47 hr. later
8	866	0.0	852	0.4	0.95 hr. later
14	1,482	1.0	1,507	1.4	no record in 6 experiments; 1.2 hrs. later in 8 experiments
20	2,041	20.3	2,039	30.7	3.2 hrs. later
10	1,035	1.2	1,081	15.7	0.6 hr. later
Totals and Averages					
92	9,511	13.4	11,093	18.7	1.33 hrs. later

Potassium cyanide.—After many preliminary observations on the proper strength of potassium cyanide, concentrations ranging between M/225,000 and M/750,000 were found to produce the desired amount of retardation without reducing the number of young which the mothers normally produce. Table IV includes the record

of completed experiments. Ninety-two individual experiments comprising over twenty thousand sexed young are given in this table. The average amount of retardation produced by potassium cyanide in the seventy-four experiments for which we have release records, was 1 hour, 20 minutes. This lengthening of the time to release the young is accompanied by an increase in male percentage from 13.4 in the control to 18.7 in the treated bottles. While this increase in male production is small when compared with that produced by the other drugs just mentioned, it is fairly consistent, only one series of experiments showing a reverse effect. This small difference may be due to the death of a few of the mothers in the bottle at the time of treatment and the subsequent reduction in the degree of crowding to which the remaining living mothers were subjected as compared with the controls. Apparently the animals vary greatly in their resistance to potassium cyanide, some of them being killed almost at once and others surviving to produce young. The increase in male production in the treated bottles was accomplished, then, in spite of the reduced number of animals which in some cases contributed to the final result. As indicated in the description (Banta and Brown, 1929*a*) of the general procedure followed in these sex-control experiments, only approximately one hundred young are sexed from each crowded bottle, so that the total young indicated in the table for the treated bottles is approximately the same as that in the control and is not reduced because of the smaller number of mothers producing young.

DISCUSSION

The data presented in this paper add a new link in the chain of evidence supporting a relationship between the rate of development of a female and the sex of her first brood of parthenogenetic young.

It was shown (Banta and Brown, 1929*d*) that the mothers from a crowded bottle which are to produce first-broods consisting of males release these males, on the average, 47 minutes later than sisters from the same bottle which produce females. It was further shown in this paper that there is a proportionate relationship between the amount of retardation in the release of young, produced by progressively greater crowding, and the percentage of males produced.

In another paper (Banta and Brown, 1929*e*) data were presented

to show that crowded mothers treated in such a way as to accelerate the rate of development, using the time of release of the first brood (i.e., the time of the fourth ecdysis) as an index of the rate of development, produce substantially fewer males than sisters not so treated. That this was not a specific action of the substances used was indicated by the many and diverse treatments which gave the same result.

It is now evident from the present data that the converse is also true: that certain substances which retard the rate of development influence the mothers to increased male production.

SUMMARY

Parthenogenetic females of *Moina macrocopa* reach a productive age more slowly when living under the influence of chloretone, phenyl urethane, or potassium cyanide.

Females thus retarded in their rate of development produce a much larger percentage of males among their first broods of young than untreated control females.

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STUDIES ON THE CONTRACTILE VACUOLE IN SPIROSTOMUM AND PARAMECIUM

(Twenty-six figures)

HOWARD CALVIN DAY
Department of Biology, Howard College[1]

INTRODUCTION

THIS paper is a continuation of a study begun in 1926 on the structure, function, and behavior of the contractile vacuole in Protozoa. The literature was reviewed in a former paper (Day, 1927); and quite recently a very comprehensive survey of the literature has been made by Lloyd (1928), to which the reader is referred. Since the appearance of the latter paper, Guilliermond (1929) has published an article on the plant vacuole; but as this apparently adds nothing to our knowledge of the contractile vacuole in Protozoa, it will not be reviewed herein.

MATERIALS AND METHOD

The organisms used in this study were the following species of Infusoria: *Spirostomum ambiguum*, *S. teres*, and *Paramecium caudatum*. The *Spirostoma* were obtained from what are known as the "Mud Lakes" located a few miles from the campus of Stanford University. The *Paramecia* were secured from a hay infusion started in the laboratory. Studies were made on these species also from cultures reared for laboratory use at the Hopkins Marine Station. The several cultures were kept in a thriving condition by adding regularly fresh water and occasionally a few grains of boiled wheat.

The studies were carried out exclusively upon living organisms. The forms to be studied were taken from the mass culture with the mouth pipette and placed upon a cover slip in a small drop of water. Evaporation was then allowed to proceed to a point when the organisms were restricted in movement by the surface film of the drop. The cover slip was thereupon inverted over a moist chamber. With

[1] This work was carried on at Stanford University under the direction of Professor C. V. Taylor.

this method (Taylor, 1920) observations can be made for almost any length of time and with any magnification of the microscope. A Leitz compound binocular microscope was used with 15× oculars, No. 6 high-power-dry and $\frac{1}{12}$ oil-immersion objectives.

The normal behavior of the contractile vacuole was first studied at length in each species with high-power-dry and oil-immersion objectives and also in the dark field. When this was satisfactorily established, the reactions of the organisms to conductivity water were studied, as was done in the previous study upon *Amoeba* (Day, 1927). Alizarin blue (Ehrlich) in graded dilutions was added to cultures of *Paramecia* in attempting to confirm the results of Howland (1924*b*). The latter used Grübler's alizarin blue of pre-war manufacture; but as the writer was unable to obtain similar dye, Ehrlich's was substituted. The infusorians were kept in small Syracuse watch glasses, and transfers were made with the mouth pipette.

Merotomy was performed on both species of *Spirostomum* and upon *Paramecium*. *Spirostomum ambiguum* is exceptionally favorable for studies in merotomy because of its moniliform macronucleus, which facilitates cutting nucleated or enucleated fragments. The cutting was done with fine quartz microneedles mounted in the Taylor manipulator (Taylor, 1925). Various transections were made, as will be seen from the accompanying figures. The same apparatus was employed for exerting localized pressure upon the organisms, with the exception that coarse needles were substituted to avoid mutilating the organisms.

Several opportunities were afforded for studying the excretory vacuole in *Spirostoma* which were in the process of binary fission.

Some experiments were carried out on lowering the temperature within the moist chamber in order to slow up the organisms and thus facilitate the study of the vacuole, assuming that this would not produce the functional disturbances characteristic of narcosis. The apparatus for this study consisted of an extra-large moist chamber with a small round opening in its roof to accommodate the hanging drop. About 8 in. of copper tubing of 3 mm. internal diameter was bent U-shape and fitted snugly into the chamber. Rubber tubing and a glass Y were used to connect the inlet with the cold and hot water which was siphoned from battery jars. The rate of flow was

governed by ordinary tube clamps. The temperature within the moist chamber was only approximately ascertained by taking the temperature of the water as it flowed from the copper tubing.

EXPERIMENTAL

The contractile vacuole of *Spirostomum* is located at the posterior end. An elongated canal runs from the vacuole to the extreme anterior end (Fig. 1). The formation of the main vesicle depends upon a coalescence of small vacuoles, as in *Amoeba* (Metcalf, 1910; Howland, 1924*b*, Day, 1927; and others). In *Spirostomum* small vacuoles can also be seen to come into contact with the canal throughout its entire length and to fuse with it, thereby emptying their contents (Fig. 1). This behavior can be clearly seen in transmitted light with the high-power-dry objective, but it is best observed by dark field illumination. The migration of the vacuoles toward the canal and the actual fusion is a relatively slow process. The vacuole enlarges to its full size, but just previous to the onset of systole a gradual constriction of the canal occurs just anterior to the vesicle proper. This constriction results in the complete severance of the canal from the vacuole (Figs. 2–4). The latter then rounds up somewhat, and upon contact with the ectoplasmic wall at the posterior tip of the organism a temporary pore is there formed through which the vacuole empties its contents to the outside. During this systole, the cytoplasm flows posteriorly, filling up the area previously occupied by the vacuole (Fig. 5). In the meantime the terminus of the canal has become bulged and a new vacuole has begun to form at the locus of its predecessor by a coalescence of minute droplets (Fig. 5). This fusion continues until a fairly large structure is formed, which then makes contact with the canal and fuses therewith to resume its normal appearance (Figs. 6–8).

The fusion of small vacuoles with the canal indicates that the canal and vacuoles are structurally similar. Their behavior can be interpreted on the basis of the sol-gel reversibility theory advanced by Taylor (1923). Further proof that this is the correct interpretation was obtained upon applying pressure to the canal at various loci along its length by means of coarse microneedles. The organism was pressed to the cover slip by slowly bringing the needles upward.

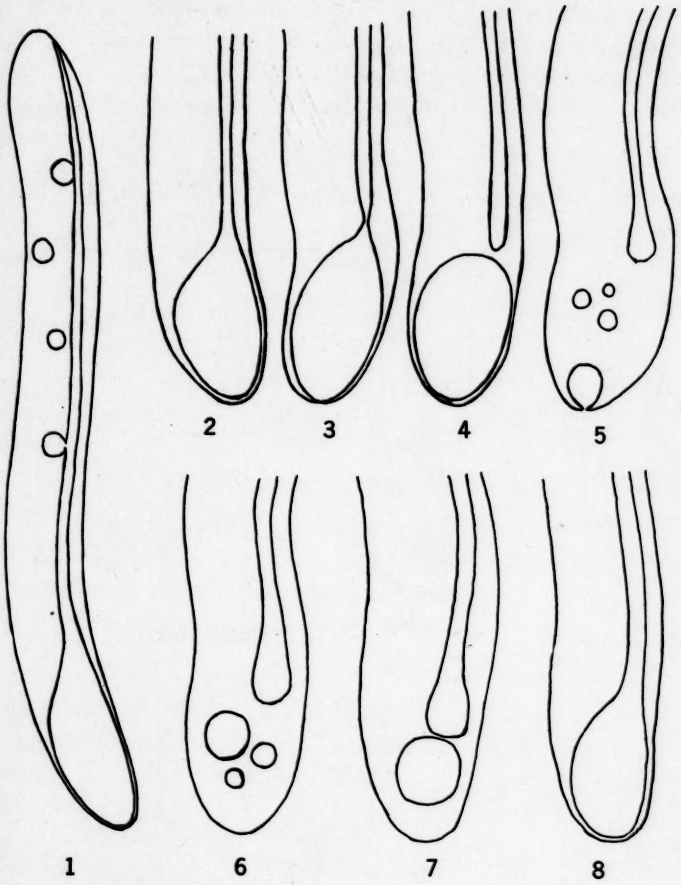


FIG. 1.—Fusion of small vacuoles with the canal.

FIGS. 2–8.—Diagrams illustrating the normal behavior of the contractile vacuole in *Spirostomum ambiguum*. Figure 2 shows the steadily enlarging vacuole; Figure 3, the beginning of the constriction of the canal; Figure 4, the complete severence of the canal from the vacuole; Figure 5, the vacuole in the process of discharge and the origin of new vacuoles; Figure 6, terminus of canal gradually enlarging and an increase in the size of the new vacuoles; Figure 7, the fusion of smaller vacuoles to form one; Figure 8, fusion of the new vacuole with canal.

On one organism local pressure was thus induced at as many as four different points, with the result that the canal was broken at these points and thereupon formed large vacuoles (Figs. 9 and 10). This can be explained by assuming that the pressure brought about a

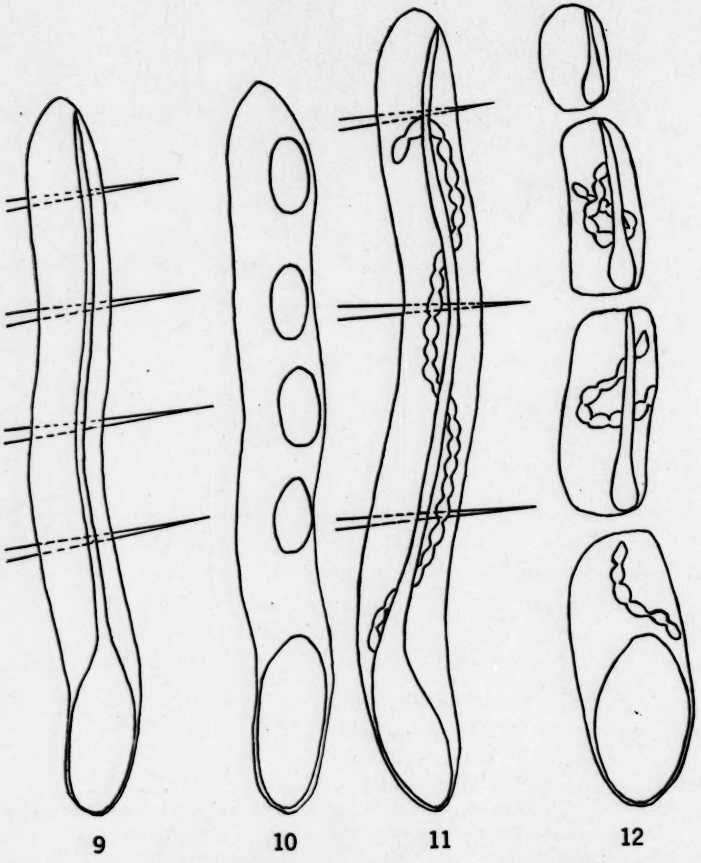


FIG. 9.—Successive applications of pressure by means of microneedles.

FIG. 10.—The resulting vacuole formation due to the effects of pressure.

FIG. 11.—Position and character of macronucleus and loci at which transections were made.

FIG. 12.—Formation of new contractile vacuoles at posterior ends of the persistent canals.

reversal of viscosity so that the gel walls became solated with a consequent rounding-up in response to the forces of surface tension. That this state was but a temporary one became evident upon isolating the organism in a small Syracuse glass: within an hour the vacuoles had again fused to form the canal.

The merotomy experiments, which were rather extensive, gave support to the theory that the contractile vacuole is a temporary structure. As noted above, a large number of transections were made anteriorly, posteriorly, and medianly. In *S. ambiguum* the transections were made so as to include portions of the macronucleus in some fragments and to exclude nuclear material in others. A few of the transections are shown in Figure 11. In all pieces, excepting the very posterior fragment, the canal terminus continued to round out; and a typical vacuolar system was formed within a half-hour following the operation (Fig. 12). The posterior piece, however, never formed a canal, although the vacuole contracted regularly. Enucleated pieces showed the same vacuole reformation as long as they survived, which rarely exceeded a few hours.

Merotomy performed on *S. teres* gave results similar to those noted above. The transections made were usually median and near the ovoid nucleus. The contractile vacuole in both the nucleated and enucleated portions reformed and functioned normally throughout the observations. A few transections through the macronucleus were attempted; but in all cases complete disintegration of the infusorians followed, so that the attempts were finally abandoned.

The same general behavior holds for organisms in the dividing state (Fig. 13). As the constriction progresses, the canal is severed and the vacuole for the anterior daughter is formed near the plane of fission, whereupon the behavior of the vacuole is identical to that observed in merotomy. The system for the posterior daughter likewise becomes a separate unit, and both vacuoles can be seen to carry on their normal functioning before the completion of fission.

In *Amoeba* (Day, 1927) it was observed that conductivity water increased the size, number, and rate of contraction of vacuoles, which led to the conclusion that the primary function of this organelle is to regulate the amount of water in the cell. By subjecting the *Spirostoma* to the same treatment, further evidence of this

function was secured. When the organisms were transferred by steps to conductivity water through a series of graded dilutions,

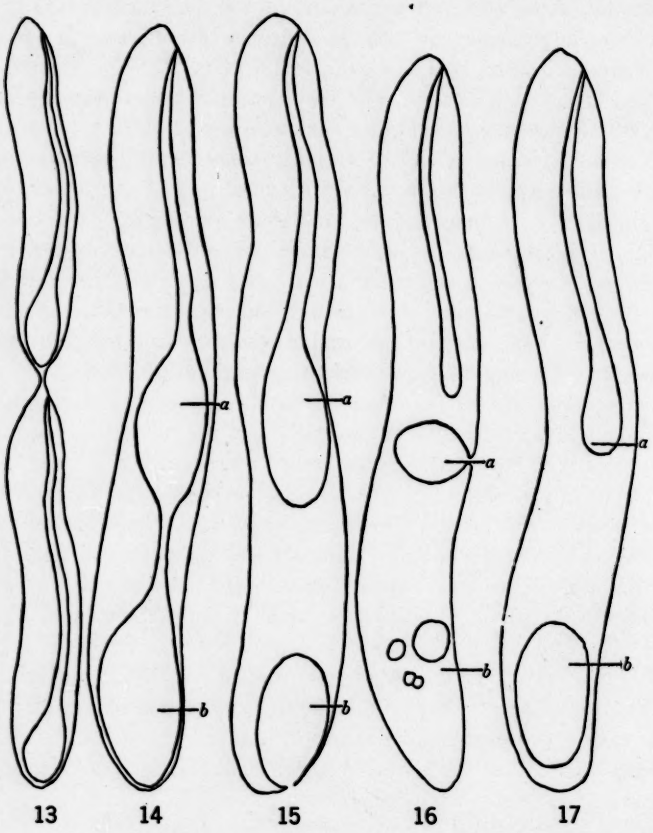


FIG. 13.—Organism in the process of fission. Note that the new contractile vacuole has been formed in the anterior daughter.

FIGS. 14–17.—Effects of conductivity water, the result of which is the formation and continuous functioning of an extra contractile vacuole.

several interesting results were obtained (Figs. 14–17). The canal bulged out into as many as four local vacuole-like swellings. In many instances a separation of the canal occurred at point *a* (Fig. 14), so

that this swollen portion became a vacuole which contracted regularly at that locus; while vacuole *b* also continued normal activity.

Similar results were obtained by adding alizarin blue (Ehrlich) in graded dilutions. This dye also apparently induces an excessive imbibition of water, with the canal reacting in the same manner as above. The action of this dye is discussed more in detail in the work on *Paramecium* reported below.

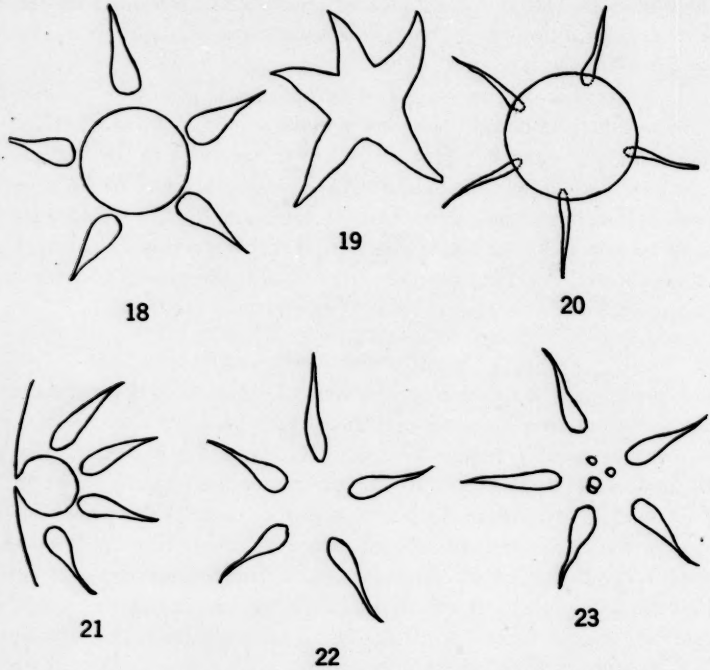
Verification of the effects of temperature gradients upon the contractile vacuole was incidentally made when temperature changes were used in lieu of narcosis, to inhibit the activity of the Protozoa. The lowering of the temperature decreases the activity of the organisms and retards the contraction of the vacuole. The temperature may be lowered to a point where total disintegration of the organisms follows. Upon raising the temperature, the rate of contraction of the vacuole is increased until a maximum is reached.

PARAMECIUM

As regards the behavior of the contractile vacuole of *Paramecium caudatum*, current opinions appear to differ widely. For this reason it seems advisable to devote special study to its behavior. Single organisms were observed with the oil-immersion objective and with dark-field illumination. It was noted that some time previous to systole the canals remain separate from the main vesicle. Then the swollen canals come into contact and fuse with the enlarging vacuole. Following the fusion, the contents of the canals are emptied into the central vacuole, whereupon they again separate from that vacuole. The vacuole in turn comes into contact with the ectoplasmic wall, where a temporary pore forms. Through this pore the vacuolar contents are discharged to the outside, and the vacuole completely disappears. For a brief time after contraction the cytoplasm that replaces the space occupied by the vacuole is optically empty. Then, near the enlarging ends of the canals there appear a few minute vacuoles which can plainly be seen to fuse with one another until a single fair-sized vacuole results, whereupon the cycle begins anew (Figs. 18–23).

When pressure is brought to bear upon the *Paramecium* by means of the microneedles, as was done with *Spirostomum*, each of

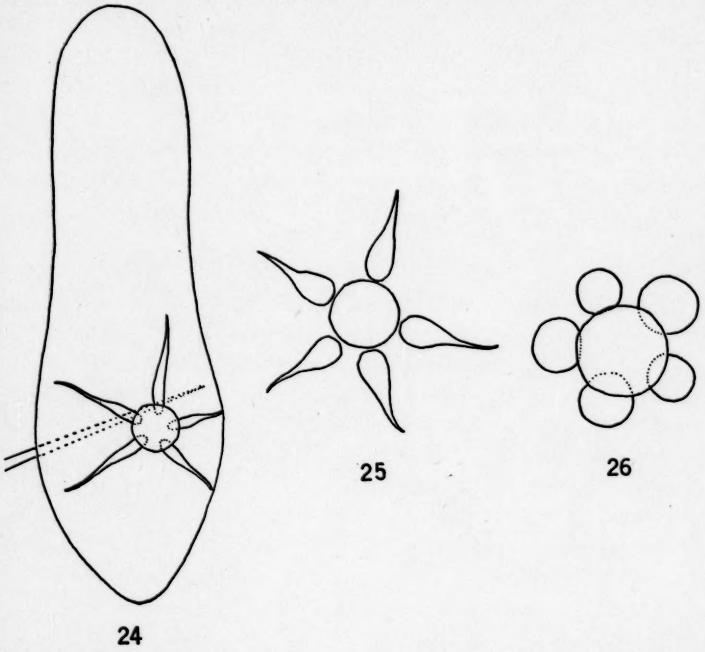
the five canals rounds up into a vacuole (Figs. 24–26). Upon releasing the pressure, the canals soon return to their normal form. Thus we see again the reversion of the gel state to that of the sol, and back again.



FIGS. 18–23.—Normal behavior of the contractile vacuole in *Paramecium caudatum*. Figure 19 shows the fusion of the enlarged canals with the vacuole; Figure 20, canals which have collapsed and the vacuole at the point of discharge; Figure 21, expulsion of vacuolar fluid; Figure 22, complete disappearance of vacuole; Figure 23, the new vacuole in the process of formation from a fusion of smaller vacuoles.

In the effort to confirm Howland's (1924*b*) results with alizarin blue, this dye was added to isolated organisms in graded dilutions. The first effect noted is the excessive intake of water, the proof of which is the greatly enlarged vacuoles. Howland records a complete gelation of the vacuole with its contributing canals to such an extent that she could cut them into pieces. In an attempt to confirm these

results, however, the organisms did not react in this manner. Instead, the vacuoles grew in size, and the canals likewise formed vacuoles in all respects identical to those caused by pressure in the experiment previously described. When a *Paramecium* was transected, the vacuoles at first floated freely in the medium, but burst



FIGS. 24–26.—Canals rounded up into vacuoles due to the effects of pressure application.

in a few seconds. Likewise, when free-floating vacuoles were pricked with microneedles, bursting followed. In neither case were there any vestiges whatever of walls or membranes.

DISCUSSION

From the experimental data submitted and a review of the literature, it appears that the contractile vacuole has at least two functions, and possibly a third. Gruber (1884), Zuelzer (1907), Hance

(1917), and others have demonstrated that vacuoles can be caused to appear where they are normally absent, as in parasitic and marine Protozoa, or to disappear from where they are normally present, as in certain fresh water Protozoa, by changing the character of the medium. These changes are brought about by altering the concentration of dissolved materials in the culture medium. The writer (Day, 1927) has shown that conductivity water, which is essentially devoid of dissolved materials, causes marked increase in the size, number, and rate of contraction of vacuoles. Howland and Pollack (1927) have increased vacuole formation by the injection of distilled water into living cells. From these results it appears evident that the contractile vacuole does perform a hydrostatic function which maintains the organism in osmotic equilibrium with its surrounding medium.

Thus far, attempts to demonstrate that the contractile vacuole is concerned with excretion have not been successful. Although Shumway (1917) and Flather (1919) attempted to show that extracts from certain endocrines increased protozoan metabolism, and hence accelerated the rate of vacuolar contractions, Woodruff and Swingle (1923) were unable to detect increases in metabolism in very careful experiments of the same nature. Howland (1924*a*) found traces of uric acid in old cultures of Protozoa by using the delicate blood-filtrate test. Weatherby (1927) and Howland (1927) found evidence of urea in culture media, but negative results were obtained when the testing-fluids were injected directly into the vacuoles. The amount of urea or ammonia, the latter formed by the hydrolysis of urea, is in no wise comparable to the volume of fluid ejected by the contractile vacuole. Thus it will be noted that, although the presence and increase of nitrogenous compounds in the culture media has been demonstrated, it has yet to be proved that these substances are excreted by the contractile vacuole.

It has been maintained that the contractile vacuole functions as an organ of respiration. Fiszer (1886) reported an *increase* in the rate of contraction of the vacuole when the concentration of oxygen was decreased. On the contrary, Degen (1905) showed that the rate of contraction *decreased* when oxygen was decreased. And Rossbach (1872) claimed that the concentration of oxygen had no effect on

the rate of vacuole contraction. These contradictory results indicate the need of further experimental data before positive statements can be made.

Nassonov's (1924) conclusion that the contractile vacuole is homologous to the Golgi apparatus of Metazoa is based chiefly upon similarity of staining-properties when fixed with osmic acid. This conclusion is worthy of consideration in connection with the study of vacuoles particularly, since that author is of the opinion that the vacuole is a permanent organelle. The concept of the contractile vacuole being a permanent structure dates back to the time of Ehrenberg (1838).

Beginning with Dujardin (1838) there has been marked opposition to this conception of the permanence of the contractile vacuole. It remained, however, for Wrzesniowski (1869) to make the first notable contribution to prove its temporary nature. This investigator observed that the contractile vacuole disappeared completely at systole and was reformed from a fusion of small vacuoles. Since this discovery, confirmation has been made in *Amoeba*, *Euplotes*, *Paramecium*, *Spirostomum*, and a number of other Protozoa.

It has here been shown that contractile vacuoles originate in fragments of various sizes, whether nucleated or not, and without respect to the region of the organism from which the fragment may be taken. It is also worthy of note that in cysts of *Euplotes taylori* (Garnjobst, 1928), where there is apparently a dedifferentiation of structures which may include the contractile vacuole, upon inducing excystment the contractile vacuole reappears and functions apparently normally, even though at first at a considerably retarded rate. Furthermore, the writer has demonstrated that the canal leading to the main vesicle of *Spirostomum* can be broken up into vacuoles by the simple expedient of applying pressure. This also holds true for the canals of *Paramecium*. These facts, together with the observation that small vacuoles fuse with the canal in *Spirostomum*, are indicative of structural similarity between the contractile vacuole and its canals.

The theory of sol-gel reversibility as applied to the behavior of contractile vacuoles by Taylor (1923) apparently applies also to the behavior of the tributary canals such as indicated in the foregoing

experiments. This behavior is strikingly illustrated both in *Spirostomum* and *Paramecium*, where, by means of pressure, the canals can be broken up into vacuoles which, upon releasing the pressure, may fuse again to form canals. Since it appears then that the contractile vacuole is a temporary structure in a considerable number of Protozoa, its behavior remains to be demonstrated in other of these unicellular organisms. It seems reasonable to suppose that vacuolar systems in all Protozoa will eventually be shown to be similar in structure and function.

In this connection, mention should be made of Howland's (1927) theory relative to the rôle of gelation in vacuolar contraction. This author concludes that vacuoles are supported in gelated areas and that the gelation initiates the contraction. Fiszer (1886) held that the pulsation of the vacuole was due to the contraction of adjacent endoplasm. In a former paper (Day, 1927) it was shown that in multi-vacuolated *Amoebae*, a condition induced by the conductivity water, as many as three vacuoles may contract simultaneously. In these organisms in which fairly large vacuoles numbered at times more than a dozen, there was normal activity. This fact is difficult to interpret if we are to assume each vacuole as being supported by a gelated area. It should also be mentioned that there was not a cessation of Brownian movement in the vicinity of vacuoles in these *Amoebae*. The writer would further call attention to the behavior of the contractile vacuole in *Euplotes* (Taylor, 1923). In this infusorian the new vacuole is formed by a fusion of about four smaller structures. During this fusion, however, the vacuole maintains a very irregular shape, as though the adjacent cytoplasm were gelated. When the coalescence is completed, the vacuole then gradually becomes round, thus indicating a solation rather than a gelation of the surrounding protoplasm.

The origin of vacuoles is still quite obscure. It is known that the immediate origin is from a fusion of minute "droplets." These may arise, as von Mohl first suggested in his study of plant vacuoles, *de novo;* or, they may arise from a coalescence of ultramicroscopic droplets; or, they may originate upon dissolution of *Exkretkörner* (Schewiakoff, 1894). Whether the origin of vacuoles in plant cells is comparable with that in Protozoa cannot at present be stated for

want of adequate data. Due caution should accordingly be exercised in making such comparisons. Exhaustive observation on a number of Protozoa with dark-field illumination, which is especially favorable for the study of vacuoles, reveals merely that vacuoles arise from a fusion or coalescence of ultramicroscopic droplets (Day, 1927). From Weatherby's (1927) observations we may conclude that soluble metabolic waste may make up a very small percentage of the vacuolar output. The remainder presumably represents water which has been taken into the organism during its feeding and by diffusion through its permeable pellicle. It is conceivable that these ultramicroscopic droplets, as previously suggested, may arise from the dissolution of *Exkretkörner*, thereby forming minute vacuoles which later fuse to form the contractile vacuole. The observations of Schewiakoff (1894), Khainsky (1910), and Taylor (1923) tend to support such an hypothesis.

SUMMARY

1. The contractile vacuole is a hydrostatic organelle which functions also in the elimination of metabolic waste.

2. Conductivity water increases the size, number, and rate of pulsation of vacuoles.

3. Alizarin blue causes excessive imbibition of water, which results in the formation of extra-large vacuoles. It further induces sol-gel reversion of the canals (*Paramecium*), which round up into spherical vacuoles.

4. Contractile vacuoles arise in nucleated and enucleated fragments of *Spirostomum* following merotomy, which indicates that this organoid is a temporary structure not affected by nuclear organization.

5. Pressure applied to the canals of *Spirostomum* and *Paramecium* causes these structures temporarily to revert to the sol state, in which there is a rounding up into spherical vacuoles.

6. Lowering of the temperature of the culture medium of *Spirostomum* and *Paramecium* slows the organisms and retards the rate of contraction of the vacuole; raising the temperature has the opposite effect.

7. Vacuolar fluid gets into the elongated canal of *Spirostomum*

by a fusion of small vacuoles with the canal throughout its entire length.

8. There is a complete disappearance of the vacuole with each contraction in *Spirostomum* and *Paramecium*.

9. The contractile vacuole in *Spirostomum* and *Paramecium* has its immediate origin in a fusion of other minute fluid vacuoles.

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THE PERMEABILITY OF COLLODION MEMBRANES IMPREGNATED WITH A PROTEIN OR WITH A LIPOID[1]

(Nine figures)

KENNETH S. RICE
Department of Physiology, University of Maine

INTRODUCTION

ADOLPH (1925) has shown that when water and 0.01 M. potassium ferrocyanide are allowed to diffuse through a membrane impregnated with a protein, the inflow of water into the potassium ferrocyanide is markedly more rapid than through the same membrane before impregnation. He ascribes this "electrostatic diffusion" to the localization at the pores of the membrane of the electrical charges borne by the diffusible ions, and points out that a plain collodion membrane cannot be considered merely from the mechanical point of view of an imperfect ion sieve but that, under certain conditions of pore size, such a membrane can itself be a localizer of charges at its pores and so exert a characteristic electrostatic effect.

It has seemed desirable to secure further quantitative evidence as to the migration of the salt through such a membrane whose nature could be experimentally modified. Accordingly a technique has been utilized which provided for the measurement of the water migration by the manometer method and at the same time for measurement of the salt migration in terms of the changing electrical conductivity of the solution on each side of the membrane. Since it has seemed advisable to use a large membrane surface in order to bring out the effect of such a porous surface, it has been necessary to devise a cell which would provide for the several measurements involved. Diagrams of the cell in essential detail are given in Figure 1.

Conductance data were obtained by balancing the resistance of

[1] Taken from thesis submitted to the department of biology in Brown University in partial fulfilment of the requirement for the degree of Doctor of Philosophy.

the cell against a known resistance by the Wheatstone bridge method. The usual set-up for conductance work was employed, and

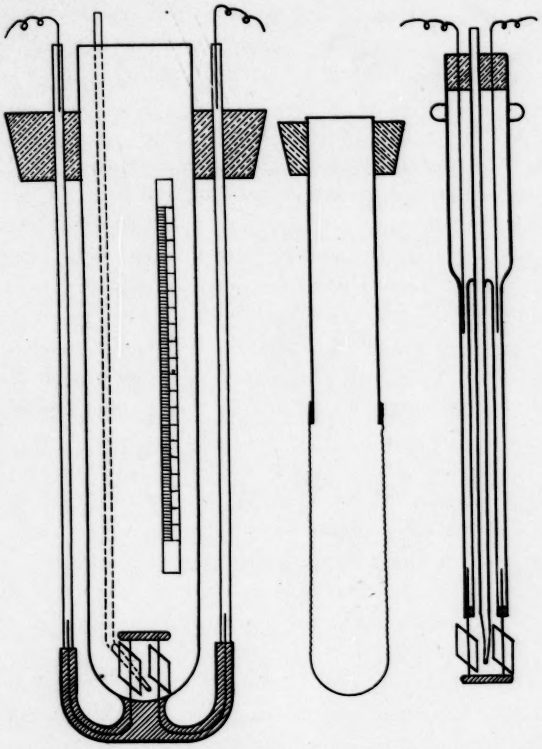

FIG. 1.—Main cell: inside diameter 32 mm., length overall 21 cm., electrodes 1 cm. sq. and 1 cm. apart; millimeter gauge on outside of cell; stirring tube sealed into main cell.

Sleeve and bag: stopper fits main cell; inside diameter of sleeve 18 mm., outside diameter of sleeve 20 mm.; inside diameter of bag 22 mm.

Dip electrodes: to fit sleeve and bag; electrodes 1 cm. sq. and 1 cm. apart.

cell constants were determined by the method of Kraus and Parker (1922). Conductance figures were obtained by the formulas given by Creighton (1924): $C = R \cdot \frac{A}{1{,}000 - A}$ and $k = \frac{K}{C}$, where $C =$ observed resistances, $R =$ resistance, $A =$ bridge reading, $k =$ specific

conductivity, and K = cell constant. No attempt has been made to use conductivity water; only distilled water in equilibrium with the carbon dioxide content of the air was utilized. Temperature was regulated by a constant-temperature water bath of 6 gallons capacity, and maintained at $25° \pm 0.05°$ C.

PROCEDURE

A bag was carefully rinsed with the solution to be used and then filled to the height of its attachment on the glass sleeve; and the inner electrode, previously rinsed, was inserted. This arrangement provided for a drop of from 10 to 15 mm. in the outside level before the unobstructed portion of the bag was exposed to the air. The cell was carefully rinsed and filled to such a level that when the bag was placed in position the level of the inside solution was the same as on the outside. Then, with solutions of equal concentration and equal level on the two sides of the bag, the cell was supported in the water bath and brought to constant temperature. The current of nitrogen to provide for stirring was started and, at constant temperature, the regulation of the bubbling was made equal and at the desired rate in each compartment. Readings were taken of the resistance as determined through each pair of electrodes. The nitrogen current was stopped so that no bubbling occurred during the actual time of reading. When the starting-point for the inside solution was thus determined, the bag with its electrodes was removed from the cell and immediately immersed in another tube of the same solution at the same temperature to prevent any further change due to temperature or evaporation from the surface of the bag while the outer cell was being washed and filled with distilled water to the previously determined level. The cell was then brought to the desired temperature and the reading of the water level was taken, thus giving a definite starting-point for each component. This record of the starting-point of the two solutions is a very necessary figure because it gives clear evidence of any error due to inadequate washing or rinsing or any variation of dilution in making up fresh solutions from time to time. The bag was then withdrawn, the accumulated drop clinging to the bottom was removed with a bit of clean filter paper, and the bag was placed in position in the outer cell.

Particular care was taken to make sure that the bag did not touch the cell at any point and that the levels of the two liquids were equal. Record of the level and of the exact time of starting was taken, and then resistance readings and level readings were taken at definite intervals on each side of the bag. At the end of a series the rate of stirring was again determined. In every case a series of records was taken of the diffusion through a plain bag, and then the same bag was impregnated in the appropriate manner and a further series recorded, thus giving a direct comparison of the plain and treated condition of the same bag. The duration of each diffusion was never less than half an hour, for experience showed that in the treated bags the difference in levels continued to increase throughout that period and reached a turning-point in just about that time. The readings of the levels were reliable within an error of ± 0.5 mm. The resistance reading of the outer solution was taken at 4 minutes past the starting-time, and that of the inner solution at 5 minutes past the starting-time. Subsequent readings on each side were taken at 5-minute intervals. Changes in the outside solution where salt was entering the distilled water took place so rapidly that it was found impracticable to balance each reading with the bridge, but experience showed that variations of resistance within 1 ohm could be determined with certainty.

In the construction of the bags care has been taken to follow the same procedure in each manipulation. The collodion suspension was made from 6 grams collodion cotton (Pyroxylin), J. T. Baker Chemical Company; 50 cc. ether, U.S.P.; and 60 cc. alcohol, 95 per cent. The method of preparation was a modification of those used by Adolph (1925). A clean, dry test tube of appropriate size was used as a mold. The tube was filled with collodion, which was poured out slowly, the mold being revolved by hand so that all the surface was thoroughly covered. When emptied to the condition of dripping, the mold was supported vertically with mouth down and allowed to remain unobstructed for 5 minutes. It was always supported at the same height and in the same location in the room to avoid any large variation in air currents.

At the end of 5 minutes of air drying, the mold was inverted and filled with distilled water. Within 5–10 minutes the water was

poured out, and the bag was withdrawn from the mold and attached to the glass sleeve. The bag fitted easily over the end of the sleeve and extended on the glass for about half an inch. This junction was then completely covered with more collodion, which served to make an adequate attachment. Then the bag was filled to the edge of the glass sleeve with water and immersed in water to the same point,

TABLE I*

SUCCESSIVE DIFFUSIONS THROUGH PLAIN COLLODION BAGS
(0.01 M. potassium ferrocyanide inside bag:
Distilled water outside bag: levels equal at start)

	Elapsed Time (Min.)	Bag 33		Bag 35		Bag 37	
		A	B	A	B	A	B
		k	k	k	k	k	k
Solution inside bag	0	50.66	50.76	50.66	50.66	51.13	51.33
	5	40.10	41.40	40.96	40.96	41.40	41.40
	10	37.38	38.50	37.38	37.74	38.12	38.12
	15	34.68	35.32	34.37	34.68	35.32	35.32
	20	32.35	32.90	31.56	32.35	32.90	33.19
	25	30.55	30.80	29.61	30.08	30.80	31.05
	30	28.52	28.95	27.90	28.10	29.39	29.39
Solution outside bag	4	2.64	2.64	3.09	2.90	3.09	3.09
	9	5.20	5.71	6.27	5.95	6.10	6.02
	14	7.39	7.92	8.44	8.28	8.28	8.05
	19	9.28	9.87	10.31	10.13	9.96	9.87
	24	10.74	11.21	11.72	11.54	11.26	11.32
	29	11.96	12.47	12.96	12.82	12.41	12.54
		Mm.	Mm.	Mm.	Mm.	Mm.	Mm.
Difference of levels	10	2	1	1	2	2	2
	20	3	2	4	4	3	3
	30	3	3	5	5	4	5

* Unless otherwise stated, all figures for specific conductance (k) are given as mhos $\times 10^4$; all differences of levels as millimeters; temperature at 25° C $\pm$ 0.05° C; stirring at the rate of 95–100 bubbles of nitrogen in 30 seconds.

allowing the junction area to dry in the air. At no time in its subsequent treatment was the bag allowed to dry out in the air beyond the very short time necessary for rinsing and filling it and changing its position.

EXPERIMENTAL

1. DIFFUSION OF 0.01 M. POTASSIUM FERROCYANIDE AND DISTILLED WATER THROUGH PLAIN COLLODION BAGS

Table I presents the records of successive diffusions through three different bags and is given in tabular form to show more clearly the

method of recording and the possibility of duplication between successive diffusions through the same bag as well as the comparison between diffusions through different bags. It is evident that, as diffusion proceeds, the conductance of the inside solution decreases at a gradually diminishing rate, while the conductance of the outside solution increases at a gradually diminishing rate in accordance with the migration of the salt. The difference in the levels recorded at any one time indicates that water is entering the inside solution at a characteristic rate. The duplication of the inside readings is excellent, but the outside readings show a greater variation, owing primarily to the extreme rapidity of the changes involved. A definite and accurate reading of the observed resistance can be obtained, but the great initial dilution of the outside solution increases the proportional rate of change of concentration so much that it is extremely difficult to time such a reading accurately at the desired interval. The duplication is sufficiently close, however, to demonstrate that, within certain limits, a given bag may be used for successive diffusions of the same electrolyte without serious complications arising from washing or rinsing.

Comparison of the three bags shows surprisingly close agreement, although no special effort has been made to standardize the bags to any definite degree of permeability. It is significant, however, that in bags 35 and 37 the manometer records showed an increased intake of water during the last diffusion of each series. This is in agreement with other records which showed that the electrolyte itself modifies the nature of a plain bag.

Subsequent records will be given in graphic form whenever possible. In all diffusions there has been no significant difference between the records for the plain and those for the treated condition of the same bag in the case of the solution inside of the bag. Accordingly, the data for the inside solutions will be omitted, although in every case the diffusions were followed by conductance readings on each side of the bag.

a) *Effect of 0.01 M. potassium ferrocyanide on a plain collodion bag.*—Bag 35, recorded in Table I, which showed a tendency toward a more rapid intake of water in the last diffusion of the series, was kept in 0.01 M. potassium ferrocyanide for 7 days; and the diffusion

record (35 A) then showed a marked increase in the rate and amount of water intake.

To verify these findings, another bag, 36, was made and kept in potassium ferrocyanide from the start. The electrolyte was used for hardening the bag, and no water was in contact with the bag until

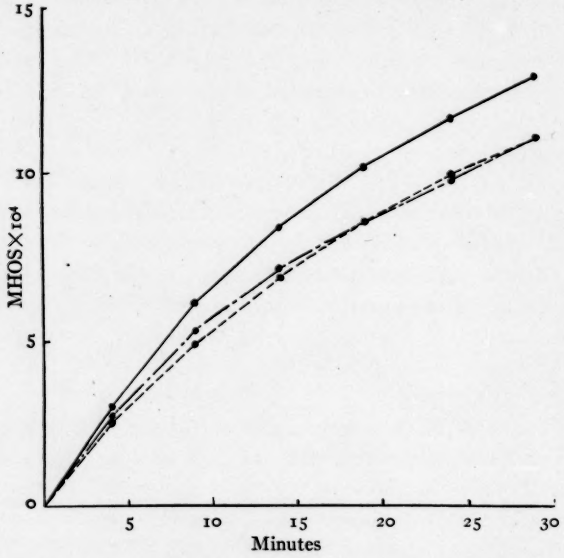

FIG. 2.—Effect of the electrolyte on plain collodion bags. Conductance records of solution outside the bag.

———— Bag 35, plain collodion.
- - - - - - - Bag 35 A, same bag kept in electrolyte 7 days after first diffusion.
—·—·— Bag 36, kept in electrolyte from the start.

the actual diffusion, when the records showed a marked increase in water intake which agreed well with the results obtained with bag 35 A. No significant change appeared in the conductance readings of the inside solution for either bag 35 A or bag 36, but in each case the conductance of the outside solutions showed a slower rate of change than that recorded for bag 35. These records are incorporated in Figures 2 and 2A. Other bags kept in distilled water for long periods showed no such results, and it is reasonable to at-

tribute the effect to an action of the electrolyte rather than to any change inherent in the bag itself.

b) *Effect of stirring.*—In the earlier series of diffusions all attempts at artificial stirring were avoided carefully on the basis that

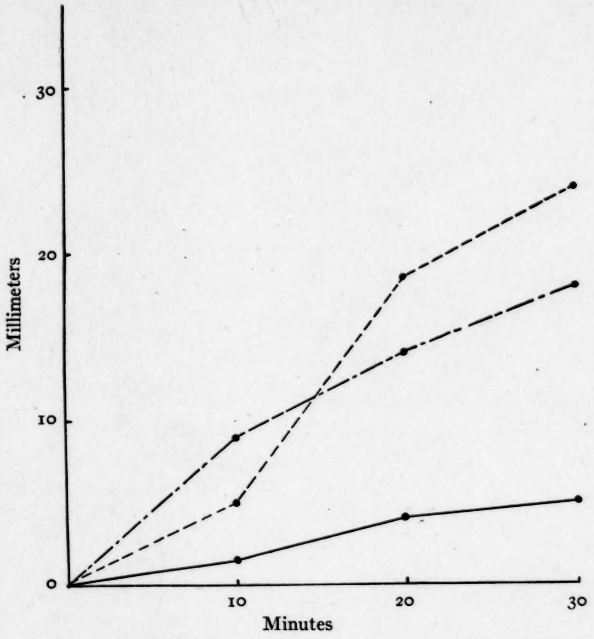

FIG. 2A.—Effect of the electrolyte on plain collodion bags. Difference in levels of solutions inside and outside the bag.

———— Bag 35, plain collodion.

- - - - - - - Bag 35 A, same bag kept in electrolyte 7 days after first diffusion.

—·—·— Bag 36, kept in electrolyte from the start.

the stirring itself would constitute a causative factor in the migration of the ions and thus would introduce another variable requiring rigid control. Further comparative data showed that very good duplication could be obtained in the conductance records of the inside solution either with or without stirring, and that closer duplication could be obtained in the records of the outside solution when no stirring was used. With no stirring used, the rate of change in the

outside solution was so rapid that accurate timing of the readings was extremely difficult, and any acceleration of this rate of change would only increase such errors. The rate of change, however, was considerably more irregular in both outside and inside solutions when no stirring was employed.

When comparison was made between diffusions through a plain bag before and after treatment, it was found that, when no stirring was used, the effect of treatment was definitely shown in the case of the outside solution but was apparently negligible in the case of the inside solution. The difference, however, was rather small; and, in view of the uneven rate of change in the inside solution obtained with a plain bag, it seemed questionable to state definitely that no change had been caused by treatment. Some method of proportionally magnifying the rate of change was needed, and stirring offered the best opportunity. Subsequent diffusions obtained when a stirring device was used gave unequivocal findings as to the effect of treatment, and are reported in detail below.

No attempt has been made to evaluate the effect of stirring in absolute terms, since no evidence is available, in the technique employed, to differentiate between the normal mobilities of the ions and the various forces, including stirring, which influence these rates of movements. Particular effort has been required, however, to maintain the stirring as a constant factor.

2. DIFFUSION THROUGH A COLLODION BAG IMPREGNATED WITH A PROTEIN

The method of impregnation was similar to that used by Loeb (1919–20) and Adolph (1925). The bags were filled to a point above the attachment on the sleeve and immersed to the same point in a 1 per cent suspension of gelatin. The time of exposure varied from 1 to 48 hours, and no particular difference in the results could be assigned to a variation in this time. Immersion in ferric chloride stained the protein treated membranes a brown color.

EXPERIMENTAL

Figures 3 and 3A present the data obtained from a series of diffusions with and without artificial stirring. In either case, however, the comparison is made between the plain and treated condi-

tion of the same bag; and in each bag the rate of change in the outside solutions is definitely slower after treatment than in the same bag before treatment. The manometer records show the characteristic acceleration of water intake through the treated bags.

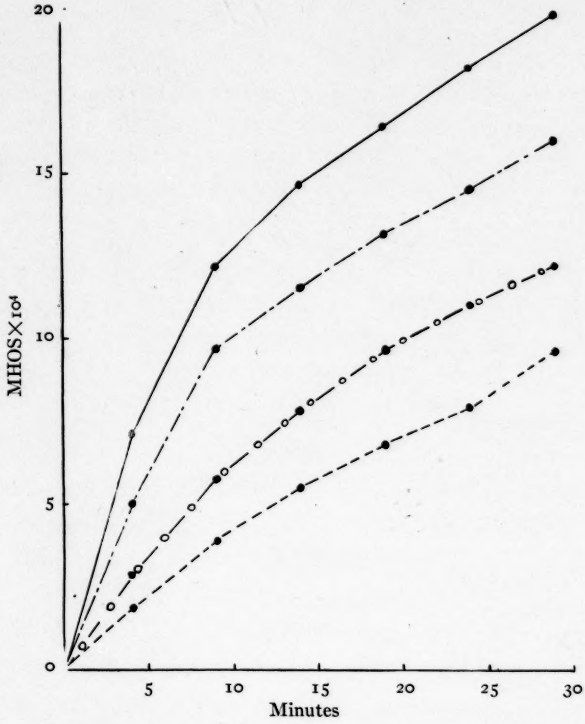

FIG. 3.—Effect of protein treatment. Conductance records of solution outside the bag.

Bag No. 2, not stirred:
———— Plain
—·—·—·— Treated

Bag No. 39, stirred:
-o-o-o-o-o Plain
- - - - - - - Treated

3. DIFFUSION THROUGH COLLODION BAGS IMPREGNATED WITH A LIPOID

That the presence of lecithin and other lipoids distinctly modifies the permeability of artificial membranes has been shown by

record (35 A) then showed a marked increase in the rate and amount of water intake.

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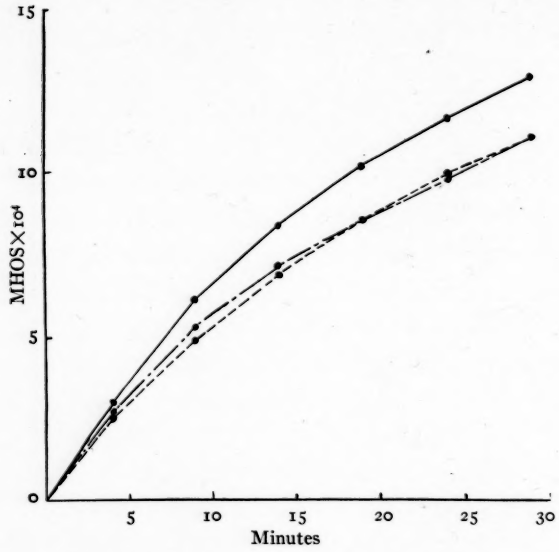

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_____ Bag 35, plain collodion.
- - - - - - - Bag 35 A, same bag kept in electrolyte 7 days after first diffusion.
—·—·— Bag 36, kept in electrolyte from the start.

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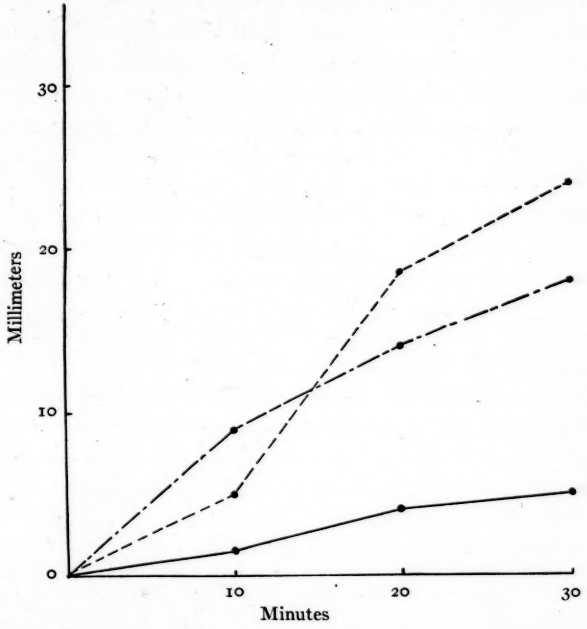

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EXPERIMENTAL

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tion of the same bag; and in each bag the rate of change in the outside solutions is definitely slower after treatment than in the same bag before treatment. The manometer records show the characteristic acceleration of water intake through the treated bags.

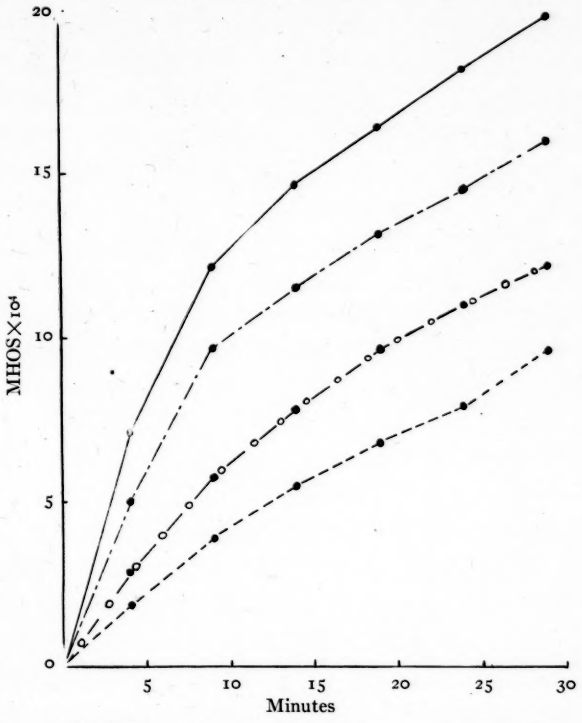

FIG. 3.—Effect of protein treatment. Conductance records of solution outside the bag.

Bag No. 2, not stirred:
———— Plain
—.—.—. Treated

Bag No. 39, stirred:
-o-o-o-o-o Plain
- - - - - - - Treated

3. DIFFUSION THROUGH COLLODION BAGS IMPREGNATED WITH A LIPOID

That the presence of lecithin and other lipoids distinctly modifies the permeability of artificial membranes has been shown by

several investigators, including Pascucci (1905) and Philippson (1913), reported by Bayliss (1920). Ruhland (1909) reported that lecithin membranes were completely impermeable until saturated with water, and Fourneau and Vulquin (1918) found that collo-

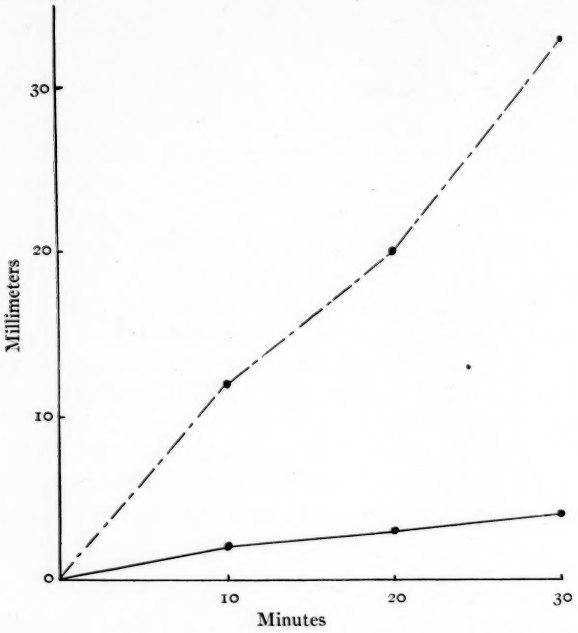

FIG. 3 A.—Effect of protein treatment. Difference in levels of solutions inside and outside the bag.

Bag No. 2, not stirred:	Bag No. 39, stirred:
———— Plain	Results duplicated
—·—·— Treated	bag No. 2

dion membranes impregnated with lecithin and with cholesterol were impermeable to electrolytes. Thieulin (1920), using 7 per cent castor oil or 15 per cent lecithin in 5 per cent collodion, made a membrane impermeable to NaCl even after 7 days of dialysis.

Unquestionably the presence of lecithin in an artificial collodion membrane does modify its permeability, and its characteristics of an

amphoteric electrolyte would suggest a possible effect related to that of the proteins. Consequently, it has seemed desirable to ascertain if the presence of lecithin alone would produce an electrostatic

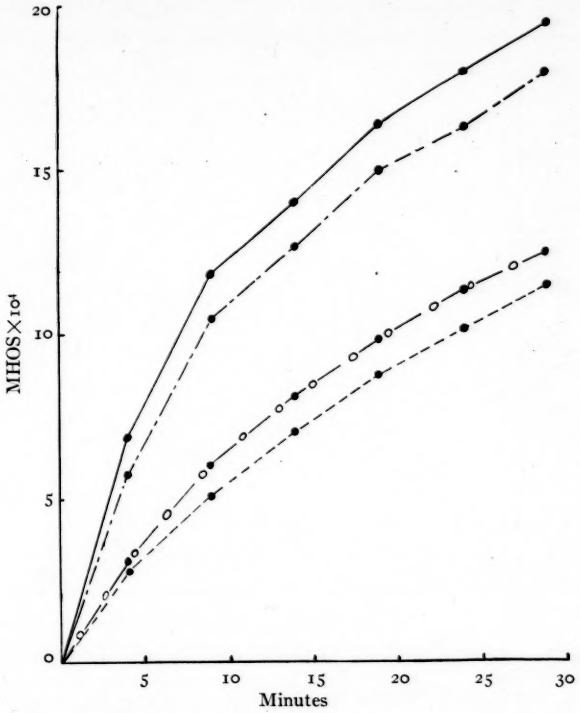

FIG. 4.—Effect of lecithin treatment. Conductance records of solution outside the bag

Bag No. 3, not stirred:		Bag No. 37, stirred:	
————	Plain	-o-o-o-o-o	Plain
—.—.—.	Treated	- - - - - - -	Treated

diffusion of water of a magnitude at all comparable to that obtained with a protein as reported by Adolph (1925) and further demonstrated in this paper.

PREPARATION OF LECITHIN

To avoid any complications due to oxidative changes, the lecithin used in this investigation was freshly prepared from brain

tissue by a method chosen from the several processes discussed by Maclean (1918). No attempt was made to secure a maximum yield; but the final product could be considered as nearly free from protein, cholesterol, and fat and as consisting of dehydrated lecithin having traces of kephalin, sphingomyelin, and cuorin. Care was taken to

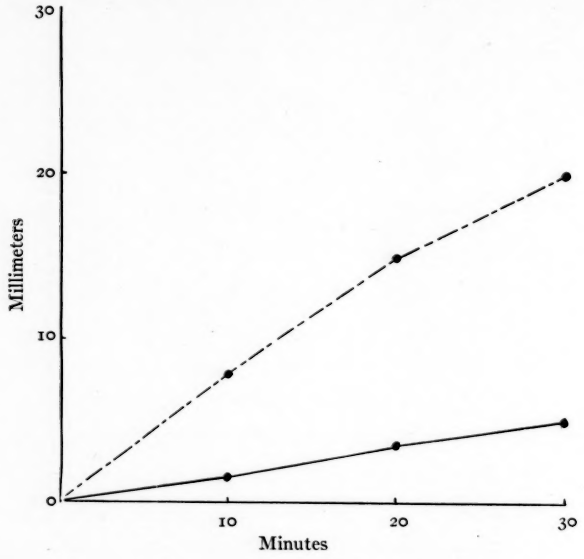

FIG. 4A.—Effect of lecithin treatment. Difference in levels of solutions inside and outside the bag.

Bag No. 3, not stirred:
———— Plain
—·—·—· Treated

Bag No. 37, stirred:
Results duplicated bag No. 3

prevent further oxidation by keeping the lecithin in a darkened vacuum desiccator.

The method of impregnation of the bags was similar to that used in the case of protein, namely, immersion in and filling with a 1 per cent suspension of the lecithin in water. Efforts to detect the presence of lecithin on the bags by chemical analysis for phosphates after such treatment were unsuccessful, but the effect on diffusion through the treated bags was unmistakable.

Figure 4 and 4A present the data for diffusion through a collodion bag before and after treatment with lecithin—bag 3, when no stirring was used; and bag 37 in which stirring was employed. Records for two bags are given and the results are essentially in agreement with those obtained with protein bags and reported in Figures 2 and 2A.

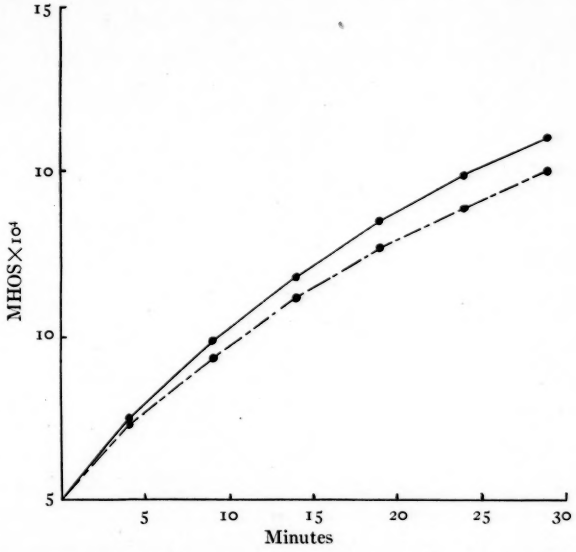

FIG. 5.—Effect of lecithin treatment on bag previously showing an effect due to the electrolyte. Conductance records of solution outside the bag.

Bag 35, kept in electrolyte 7 days:

———— Plain

—·—·—· Treated

a) Effect of lecithin treatment on bags previously showing an effect due to the electrolyte.—To make sure that the effect of the lecithin treatment was independent of any action of the electrolyte, bags 35 and 36, which had demonstrated the "electrolyte" effect and are reported in detail in Figures 1 and 1A, were impregnated with lecithin. In each case the manometer records showed an increased intake of water; and the conductance records of the outside solutions showed a retardation in rate of change beyond that induced by the

exposure to the electrolyte, while the inside conductance records showed no certain variation.

Figures 5 and 5A present the records of bag 35. Bag 36, having

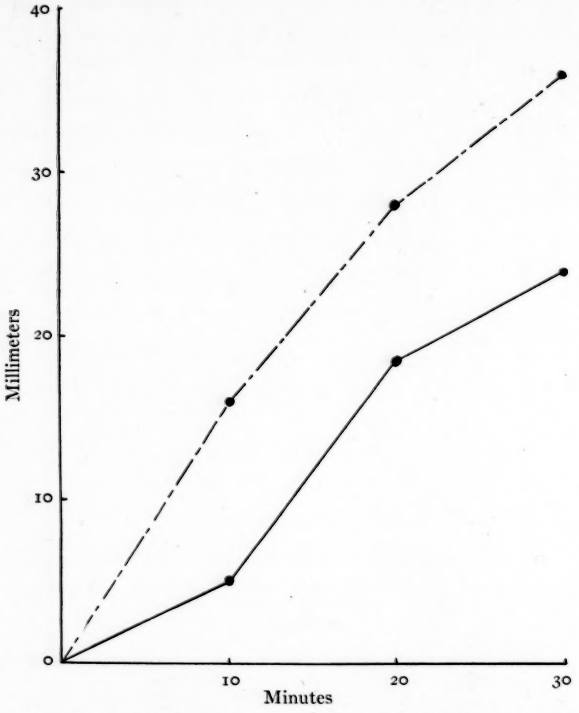

FIG. 5A.—Effect of lecithin treatment on bag previously showing an effect due to the electrolyte. Difference in levels of solutions outside and inside of bag.

Bag 35, kept in electrolyte 7 days:
———— Plain
—·—·—· Treated

a slightly longer exposure to the electrolyte, gave results essentially similar to bag 35 and has not been reported separately.

b) *Effect of water on lecithin-treated bags.*—To test the possible washing-out effect of water, bags which had been treated with lecithin were kept in distilled water for various lengths of time and are reported in Table II. Bag 31, which had previously shown the

typical lecithin effect, was held in water for 24 hours and then showed a marked loss of the lecithin effect. After continuing the washing for 4 days, the water intake was comparable to that of the bag before treatment, and the conductance record of the outside solution showed nearly the same rate of change as that of the plain bag. In all cases, there was no significant change in records of the inside solutions.

TABLE II

EFFECT OF WATER ON LECITHIN-TREATED BAG
BAG 31—STIRRED

	Elapsed Time (Min.)	Plain	Treated	Washed 24 Hours	Washed 4 Days
		k	*k*	*k*	*k*
Solution outside bag	4	2.9	1.7	2.6	2.7
	9	5.5	3.9	5.0	5.4
	14	7.5	5.7	7.1	7.3
	19	9.2	7.2	8.7	9.0
	24	10.5	8.5	10.0	10.4
	29	11.7	9.5	11.1	11.5
		Mm.	Mm.	Mm.	Mm.
Difference of levels	10	3	13	7	4
	20	4	23	12	7
	30	5	30	16	9

DISCUSSION

From the data thus far reported it seems evident that, under the experimental conditions and within certain limits, successive diffusions of potassium ferrocyanide and distilled water through the same collodion bag can be reproduced with reasonable accuracy; and similar diffusions through several bags made by the same technique show close agreement. Further exposure to the electrolyte produces modifications of the bag attributable to the action of the electrolyte.

Constant stirring of the solutions on each side of the bag produces a much more constant rate of change.

The difference between the levels of the two solutions at definite intervals can be taken as an accurate measure of the rate and amount of water migration, but such records give no quantitative evidence as to the comparative value of the various forces responsible for the movement observed.

The changes in conductance figures obtained in each solution are

due to the change in the concentration of the available electrolyte, and as such can be taken as an accurate measure of the rate and amount of salt migration through the membrane; but here again, the figures represent the net result of several forces whose separate values cannot be determined by this technique.

In the case of the protein-treated bags, it is clear that water enters the treated bag much more rapidly than through the same bag before treatment, which agrees with the results obtained by Adolph (1925); and further evidence is presented in the conductance figures to show that the salt migration is definitely retarded through the treated bag.

Similar results are obtained both as to water and salt migration when the bag is treated with lecithin.

Further analysis of the conductance data shows that the forces responsible for the retardation of the salt migration through the treated bags are approximately equivalent to the forces responsible for the acceleration of the water movement. In the case of the treated bags, the more rapid intake of water would be expected to produce a marked dilution effect; but such dilution was apparently offset by an equivalent slowing of the salt migration, and this balance of forces is evidenced by the consistent duplication of the conductance figures obtained in the solutions inside the bags both before and after treatment.

The lecithin treatment is much less permanent than the protein treatment and is gradually reduced by subsequent washings, while the protein-treated bags show the characteristic effect even after repeated washings.

SUMMARY

A method is presented for the quantitative determination of the rate and amount of migration of both salt and water occurring in diffusions of 0.01 M. potassium ferrocyanide and distilled water through collodion bags before and after treatment with a protein and with a lipoid.

The water migration is given in terms of millimeters of difference of levels of the solutions inside and outside of the bag, and the salt migration is expressed in terms of the difference of the specific conductance of the two solutions.

Both protein and lecithin treatment of a collodion bag induce a definite acceleration of water migration and a retardation of salt migration as compared with the rate of movement through the same bag before treatment.

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THE SUSCEPTIBILITY OF PLANARIA TO POTASSIUM CYANIDE IN RELATION TO HYDRION CONCENTRATION AND TO CERTAIN CHANGES IN SALT CONTENT OF THE MEDIUM

(Thirteen figures)

C. M. CHILD
Zoölogical Laboratory, University of Chicago

DURING the period in which *Planaria* has been extensively used as experimental material in this laboratory, a considerable volume of data concerning the susceptibility of this animal to various agents under various conditions has accumulated. In many cases the original data have been confirmed by the observations of students repeated year after year.

In the course of study of susceptibility it has been found, as might be expected, that the susceptibility of *Planaria* to a given concentration of KCN varies very greatly with hydrogen-ion concentration and salt content of the solution. The purpose of the present paper is to record some of these differences in susceptibility.

MATERIAL AND METHODS

Planaria dorotocephala has served as the chief experimental material but has been supplemented by *P. maculata*. Since the latter species is not known to occur in the vicinity of Chicago, stocks have been obtained from Wood's Hole and New Haven; but the relatively small number of animals in such stocks and the needs of other lines of investigation have necessitated some limitation in the use of this species as material for the study of susceptibility. In general smaller numbers of animals have been used in each lot, and the series of experiments with *P. maculata* is less extensive than with *P. dorotocephala*. But since the results do not differ greatly in the two species, the data on *P. maculata* serve to supplement and confirm those on *P. dorotocephala*.

The experiments consist essentially in determining the survival time of a certain number of individuals in a certain solution. With *P. dorotocephala* each experimental lot usually consists of ten in-

dividuals; with *P. maculata*, of five or six. For each lot animals of the same length which have not recently undergone fission are selected from the same stock, all members of which have been kept in the same container. In most of the experiments on *P. dorotocephala* "full-grown" animals approximately 20 mm. in length have been used. Such animals are full grown as regards the anterior zooid. The total body length may become much greater than 20 mm., but all or practically all such increase in length occurs postorally in the posterior zooid region, which may become two or three times as long as the preoral region. The comparative data on susceptibility concern only the first zooid, i.e., the region between the head and the level at which fission occurs 2–3 mm. posterior to the mouth; consequently the number and length of the posterior zooids in these large animals make little difference as regards these experiments. In a part of the experiments with *P. dorotocephala* recorded below, animals 6–7 mm. in length have been used for comparison with animals 20 mm. or more in length. Most of the data on *P. maculata* were obtained with individuals 12–14 mm. in length, usually in lots of five or six; but in a few cases it was necessary to use somewhat smaller animals 10–12 mm. in length.

The paper is primarily concerned with the susceptibility to m/1,000 KCN in well water, over a wide range of pH, in distilled water, and in various balanced salt solutions. HCl was used to decrease the pH of the KCN solutions, and some data on susceptibility in relation to pH were obtained with KOH and HCl solutions in well water and in distilled water for comparison with those on susceptibility to KCN at different pH.

Ringer solutions were made up on the basis of two formulas, as follows:

SOLUTION A	Grams
NaCl	6.5
$CaCl_2+2H_2O$	0.16
KCl	0.14
Distilled H_2O to 1,000 cc.	

SOLUTION B	
NaCl	2.5
$CaCl_2+2H_2O$	0.25
KCl	0.05
Distilled H_2O to 1,000 cc.	

To attain a given pH, $NaHCO_3$ is added to these solutions or dilutions of them. Solution A is essentially the Ringer solution as given by Bayliss (*General Physiology*) for the amphibian heart. Solution B is the modified Ringer found by Murray (1928) to be most satisfactory for *P. dorotocephala*.

In the determination of susceptibility by survival time for *Planaria* it is possible with most lethal agents to use the visible disintegration of the tissues, particularly of the body wall, as an indicator of death, since it is closely associated in time with death and, whether it precedes or follows death, represents an irreversible injury. When the concentration or dosage of the agent is above the limit of tolerance and yet low enough so that death does not occur at once, the death of the anterior zooid begins at the head and progresses posteriorly to the level of fission. The posterior tip of the body, a growing region, may be even more susceptible than the head; and the susceptibility of the posterior zooids differs in different individuals.

With gradual death and disintegration various measures of susceptibility are possible. In the method which was used in earlier work a number of successive periods or stages are distinguished, e.g., intact animals, period of head disintegration, disintegration of lateral margins or of anterior half of first zooid, and so on to complete disintegration of the body wall. In experiment the distribution of individuals in the different stages, including intact animals and those completely disintegrated, is recorded at regular intervals. Numerical values in definite order are assigned to each stage, e.g., 40, 30, 20, etc., the intact stage receiving the highest, the stage of complete disintegration being 0. The number of individuals in each period of disintegration at a given time is multiplied by the numerical value of that period; and the sum of these products for all the periods at that time, divided by the number of animals in the experimental lot, gives a number which may be regarded as a numerical expression of the average stage of disintegration at that time. In graphing, these numbers for successive times of observation are plotted as ordinates against times as abscissas, giving a descending curve of the course of disintegration of the first zooid from the head posteriorly. With this method of plotting, the higher the susceptibility the farther to the left the curve lies, and the more rapid the course of

disintegration the steeper the curve. This method involves following the course of disintegration from beginning to end with records at stated intervals, and in cases of disintegration extending over many hours or days it presents difficulties. Its chief advantage is that it permits comparison of the rapidity of progress of disintegration from the head posteriorly under the different conditions of experiment.

More recently, single numerical indices of susceptibility have been found to serve as well as the curves for general comparison of survival times under different conditions. For example, the number of hours from beginning of the experiment to beginning of disintegration, or from beginning of experiment to completion of disintegration, may be used, but the latter also involves following disintegration to the end. Another index of susceptibility is the time in hours from beginning of the experiment to the time when disintegration of the head has begun in 50 per cent of the experimental lot. This, the beginning time and the mean time of disintegration as defined above, and the susceptibility curves show similar differences under different conditions, with occasional slight departures from uniformity, as might be expected in different lots of ten animals each. In the following tables the beginning time is used. It is believed that this time is more satisfactory for present purposes than the mean time or the curves because under some conditions the later stages of disintegration are often complicated by the protrusion of the pharynx through the body wall, the wound thus formed becoming a region of high susceptibility. Also, in well fed animals the alimentary tract is often more susceptible than the body wall, and it may swell and break through the body wall at various points while the latter is otherwise intact. Each such injury becomes a region of high susceptibility and disintegration. And finally, under certain conditions gland cells of the lateral margins and dorsal surface show a high susceptibility and early disintegration. These complications often render the time of completion of disintegration uncertain. The beginning of disintegration in the head region, however, is free from such uncertainty.

In certain solutions, e.g., KCN m/1,000 in certain modifications of Ringer, death is not accompanied by disintegration except sometimes at the extreme anterior end and the posterior growing tip and

the bodies remain intact until maceration gradually takes place without any indication of a gradient.

Under such conditions the differential susceptibility and the course of death or irreversible toxic effect are readily determined by placing a large number of animals in the agent and by returning at stated intervals a sample consisting of a given number of animals to water. In water the parts which are killed or irreversibly injured disintegrate rapidly while other parts remain alive and later reconstitute new individuals. In this manner it can be shown with the greatest clearness that a longitudinal gradient in susceptibility to KCN-Ringer solution exists, although animals left in the solution show only slight and uncertain indications of such a gradient.

In all experiments with KCN and HCl, Erlenmeyer flasks, usually 500 cc., were used. These were filled with solution, leaving only a small air space in the neck to avoid breakage with slight change of temperature, and tightly corked. For KOH solutions and distilled water finger bowls were used.

KCN m/1,000 by weight in well water was selected as a satisfactory basic concentration. In this solution the 20 mm. animals show no indication of stimulation, but creep more and more slowly, gradually lose control of the posterior zooid region, and finally become incapable of co-ordinated movement. These effects occur in the course of 2–3 hours, and somewhat later disintegration begins at the anterior end and progresses posteriorly in the first zooid. Some muscular activity usually persists in the postcephalic region of the first zooid, even after the head is completely disintegrated. About the same time that disintegration begins in the head region, it also begins in the posterior tip of the body. In 20 mm. animals disintegration in the posterior zooid region usually indicates the presence of two posterior zooids, of which the more posterior is the more susceptible. Disintegration of the body wall is completed in 7–10 hours, the posterior end of the first zooid, a region 2–3 mm. posterior to the mouth, being the last part to disintegrate. This gradual progress of the lethal effects permits the observation of details which are less evident in higher concentrations, and KCN m/1,000 is at the same time far above the limit of acclimation or tolerance. The susceptibility of 20 mm. well-fed animals in KCN m/1,000 serves, then, as a starting-

point for the investigation of the alterations of susceptibility by changes in pH and salt content of the solution.

The pH of solutions was estimated colorimetrically with the aid of standard tubes and the Clark color chart. Exact determinations of pH have no significance, for differences even of one- or two-tenths pH alter susceptibility only slightly. It should be noted that most of the general results presented below—that is, the increase or decrease of susceptibility with certain changes in the solutions—have been repeatedly obtained by students in laboratory work as well as by the writer. The actual survival time varies with size of animal, nutritive condition, temperature, etc. The data presented below, with the exception of those on small animals in the third section of Table I, represent results obtained with animals of approximately the same size and nutritive condition at temperatures of 20°–22° C.

The experimental lots were not always under observation at the exact time when 50 per cent began to disintegrate at the anterior end. The index of susceptibility as given is very commonly estimated from conditions at two successive observation times, one with less and the other with more than 50 per cent beginning to disintegrate. In most series observations were made each hour or under conditions of high susceptibility each half hour, and the time error involved in estimation is negligible for the comparative purposes of the present paper.

SUSCEPTIBILITY TO KCN IN RELATION TO pH

Table I shows the decrease in susceptibility with decrease in pH. The first two sections of the table give data for animals 20–25 mm. in length, the first zooid in all cases being approximately half the total length. In the third section some data on small animals are given. Lots 1–8 in the first section show the range of variation in susceptibility of large animals in KCN solutions in a natural water (lake or well water) with a pH of approximately 9.2. Except for Lot 8, the index of susceptibility ranges from 3 to $4\frac{1}{2}$ hours. Lot 8 consisted of animals which had been fed three times a week during 4 months. Such animals are really overfed and are loaded with reserves and always show a lower susceptibility than those less heavily fed.

The second section of Table I shows the very marked progres-

sive decrease in susceptibility with decrease in pH to below 6.5. At 6.2 (Lots 18, 19) susceptibility begins to increase again, and at pH

TABLE I

SUSCEPTIBILITY OF *Planaria dorotocephala* TO KCN m/1,000 IN WELL WATER AT DIFFERENT pH AS INDICATED BY BEGINNING OF DISINTEGRATION IN 50 PER CENT OF HEADS

(Ten animals in each lot except Nos. 6 and 25 which consist of five each)

Lot No.	Length in Millimeters	pH	Time in Hours
1	18–20	9.2	3
2			3
3			3¼
4			3¼
5			4
6			4½
7			4
8			6
9	18–20	7.6	17
10		7	12
11		7	15
12		7	16
13		7	18
14		6.8	16
15		6.6	23
16		6.6	23½
17		6.5	22
18		6.2	14
19		6.2	13
20		5.4	3¼
21	7–8	9.2	2¼
22			2¼
23			2½
24			2½
25			3
26	7–8	7.6	13
27		7	11
28		7	14
29		6.8	13
30		6	11½

5.4 (Lot 20) it is greatly increased and about the same as in the alkaline KCN solution at pH 9.2. With further decrease the pH rather than KCN determines the susceptibility, as Table II will show.

The third section of Table I gives data on smaller, physiologically younger animals (7–8 mm.). In Lots 21–25 the index time runs from $2\frac{1}{2}$ to 3 hours. The index of 3 hours in Lot 25 is as large as that of large worms in Lots 1 and 2 of the first section, but the susceptibility of 7–8 mm. animals is always 1–2 hours higher than that of 20 mm. animals from the same stock tested at the same time. Lot 25, for example, with an index of 3 hours was from the same stock and was

TABLE II

SUSCEPTIBILITY OF *Planaria dorotocephala* IN WELL WATER BROUGHT BELOW pH 6 BY ADDITION OF HCl, AS INDICATED BY TIME IN HOURS OF BEGINNING DISINTEGRATION OF 50 PER CENT OF HEADS

(Ten animals in each lot)

Lot No.	Length in Millimeters	pH	Time in Hours
1	18–20	5.8	All live (3 heads disintegrate)
2		5.6	All live (3 heads disintegrate)
3		5.4	24 (1 lives)
4		5	5
5		4.6	3
6		4.6	$2\frac{1}{4}$
7		4.2	$1\frac{1}{4}$
8		3.8	$\frac{3}{4}$
9		3.8	$\frac{1}{2}$
10	7–8	5.8	All live
11		5.6	6 live
12		5.4	6 live
13		5.0	12
14		4.6	$3\frac{1}{2}$
15		4.6	$1\frac{1}{2}$
16		4.2	$\frac{3}{4}$
17		3.8	$\frac{1}{2}$ —
18		3.8	$\frac{1}{4}$ —

run at the same time as Lot 6 of the first section with an index of $4\frac{1}{2}$ hours. Lots 26–30 show the decrease in susceptibility with decrease in pH to pH 6. With a little further decrease pH determines the susceptibility (see Table II).

It is sufficiently evident from Table I that the alkalinity of the KCN solution in well or lake water is an important factor in determining the survival time, and that with decrease in alkalinity the susceptibility decreases until the pH itself becomes a factor in determining susceptibility.

SUSCEPTIBILITY IN HCl

Table II presents data on susceptibility at different pH as obtained by the addition of HCl to well water. The first section of the table gives the susceptibilities for large animals; the second, those for small animals. In each HCl experiment one lot of large and one of small animals from the same stock was run at the same time. In Table II the nine lots of each section taken in order represent such lots— each pair, Lots 1 and 10, Lots 2 and 11, and so on, representing large and small animals from the same stock run at the same time and under the same conditions. The differences in susceptibility in each pair are then directly comparable as differences of large and small, or physiologically old and young, animals.

Above pH 6 and usually at pH 6 all the large animals remain alive indefinitely. Below pH 6 disintegration begins to occur in some individuals. In Lot 1 of Table II at pH 5.8 three heads disintegrated (between 16 and 92 hours), but the disintegration went no farther and seven animals remained intact. In some other lots at this pH all remained intact. In Lot 2 at pH 5.6 all animals in this particular lot of ten remained intact. In some other lots at the same pH more or less disintegration of the anterior region occurred in some animals. Lot 3 at pH 5.4 shows a great increase in susceptibility, the index being 24 hours; but one animal remained intact for 120 hours, i.e., as long as observation was continued. Lot 4 at pH 5.0 shows again a great increase in susceptibility over Lot 3, the index time being 5 hours. In the following lots at still lower pH susceptibility increases still farther, until in Lots 8 and 9, both at pH 3.8, the indices are $\frac{3}{4}$ and $\frac{1}{2}$ hour.

As regards the small animals of the second section, all live without disintegration at pH 5.8 (Lot 10), six live without disintegration at pH 5.6 (Lot 11), and six at pH 5.4 (Lot 12). At pH 5 (Lot 13) the index time is 12 hours as compared with 5 hours in Lot 4, and at pH 4.6 (Lots 14 and 15) the index time is $3\frac{1}{2}$ hours and $1\frac{1}{2}$ hours as compared with 3 hours and $2\frac{1}{4}$ hours in Lots 5 and 6. In other words, from pH 5.8 to pH 4.6 the small worms appear to be somewhat less susceptible than the large. This difference is due to the greater ability of the physiologically young animals to acclimate or acquire tolerance. Acclimation to acid occurs rapidly, i.e., within a few days if it

occurs at all; and the small young animals are able to acclimate more rapidly or more completely than the large old animals; and even if they do not live indefinitely, they may live longer than the large old animals, as in Lots 13 and 14 compared with Lots 4 and 5. These differences in ability to acclimate are similar to those observed by MacArthur (1920) in his study of acclimation to acid. Further confirmation of these results has been obtained by repeated use of similar experiments in laboratory class work.

Evidently pH 4.6 represents about the lower limit of acclimation of the small animals for comparison of Lots 14 and 5, both pH 4.6, shows the small animals slightly less susceptible ($3\frac{1}{2}:3$); while comparison of Lots 15 and 6, also pH 4.6, shows the small worms slightly more susceptible than the large ($1\frac{3}{4}:2\frac{1}{4}$). With still lower pH (Lots 16–18) the susceptibility of the smaller animals is always higher than that of the large (Lots 7–9).

Table I shows that with decrease in pH of m/1,000 KCN to 6.2 and below, susceptibility begins to increase; while, according to Table II, pH alone does not become lethal until about pH 5.4. In other words, with decrease in pH from 6.2 to a point somewhat below pH 5. at which the hydrogen-ion concentration becomes the lethal factor, KCN m/1,000 is somewhat more toxic than HCl alone.

SUSCEPTIBILITY OF PLANARIA DOROTOCEPHALA TO DISTILLED WATER AND TO CERTAIN SALT SOLUTIONS

Planaria dorotocephala does not live indefinitely in distilled water, but in distilled water plus small amounts of the salts of Ringer solution it does live indefinitely. At present it is desired merely to call attention briefly to certain points in this connection which have a bearing on other observations to be considered in following sections.

The survival time of *P. dorotocephala* in distilled water differs greatly according to the number of worms of a given size in relation to volume of water, the frequency of change of the distilled water, and to some extent the number of times the animals are washed in distilled water before being left in a certain volume. It seems evident that the animals condition the water by giving off salts so that it is no longer distilled water, and, if their size and numbers are sufficient in relation to the volume, may add sufficient salts to it to permit

them to live indefinitely. The following examples will serve to illustrate the situation. Ten animals of 20 mm. size in 250 cc. distilled water without change gave an index time of 13 hours, in 125 cc. 24 hours, and with further decrease in volume or increase in number they live still longer. On the other hand, when the distilled water is frequently renewed, e.g., every hour, the survival time is only 6–8 hours for the large animals and still less for the small.

Murray (1928) has presented some data on the susceptibility of *Planaria* to salt solutions and has found that salt ratios approximating those of solution B (p. 91) (that is, high Ca and low K rather than the usual ratios of solution A) afford the most satisfactory salt medium for culture of the tissues of *P. dorotocephala*. In the experiments to be reported here, the salt ratios of the solutions A and B have been used in three concentrations each: the concentration as given (p. 91), and one-half and one-tenth of this concentration. With the object in view of determining how changes in Na, K, and Ca content and ratio alter susceptibility to KCN, the three concentrations of solutions A and B were used as follows. First, susceptibility of *P. dorotocephala* to these solutions brought to pH 7.5 by $NaHCO_3$ was determined. Second, since a KCN solution contains KOH, the effect of these solutions on susceptibility to KOH was determined. Third, susceptibility to KCN m/1,000 made up in these solutions was compared with that to KCN in well-water and distilled-water solutions. As regards the susceptibility to the salt solutions alone, it may be noted first that none of the six is rapidly lethal but that marked differences in susceptibility to them are associated with differences in physiological age and nutritive condition. In general, small, physiologically young animals (e.g., 7–8 mm. or less in length) are more susceptible to the higher concentrations and show a much greater ability to acclimate to salt solutions than the old, large animals (18–20 mm. or more in length). Young animals may be able to live indefinitely in a solution which kills the older after several days. For example, with animals from certain stocks which had been kept in the laboratory for some months all of the large animals gradually underwent complete disintegration in solution A/10, while all of the young animals in the same container remained intact. In solution A/2 laboratory stocks usually show a similar dif-

ference between old and young animals, though less marked; i.e., some of the young animals live and all of the old die, or more of the young than of the old live. This lower susceptibility of small animals in the lower concentrations of solution A is unquestionably a matter of acclimation, for the susceptibility of the small animals to sufficiently high concentrations is distinctly greater than that of the large. Animals of a given size from freshly collected stocks show a greater ability to acclimate to the salt solutions than those kept for long periods in the laboratory. In the very low concentrations susceptibility depends to some extent on volume and frequency of renewal of the solution in relation to the volume of tissue of animals. With such concentrations the susceptibility is less in a small volume of solution not renewed than in a large volume with frequent renewal.

In determining the effects of the different salt solutions, ten animals of the same size were washed once in the solution to be used and then brought into 250 cc. of the solution in a finger bowl, and the solution was not renewed. Solution A produces no visible effect at first; but in the course of a day or two the animals become incapable or almost incapable of progressive locomotion, although muscular contraction continues for days in the form of irregular bendings and contortions. In all lots observed thus far from various stocks in various condition, solution A has killed sooner or later, that is, after 4 days to a week or even more. In solution A/2 some of the old animals 16 mm. or more in length from old laboratory stocks die after 7–10 days, but animals of the same sizes from freshly collected stocks are less susceptible and often live for several weeks, probably indefinitely. Young animals, even from the laboratory stocks, are less susceptible than the old and in most cases live for several weeks, probably indefinitely. The occurrence of more or less acclimation of younger animals in solution A/10 has already been noted. All animals 18–20 mm. in length from old laboratory stocks die in 10–13 days, while animals 7–8 mm. in length from the same stocks and in the same container are intact and active after 16 days. It is possible that in such cases the death and disintegration of the large animals supplies additional salts and so makes continued life possible for the small animals; but this possibility does not alter the fact that the young animals remain intact and active while the old

begin to die after some days. Both large and small animals, newly collected, live, with occasional exceptions, for several weeks and probably indefinitely in solution A/10.

The solutions B and B/2 do not kill and are not toxic to *P. dorotocephala*, but B/10 may kill some of the larger animals of old stocks and occasionally a small animal in the course of 7–10 days, while the animals of new stocks remain intact at least for several weeks. Considering these susceptibilities in the light of Murray's work (Murray, 1928), it is evident that the calcium-potassium ratio in solution A is not suited to *P. dorotocephala*. The more or less complete paralysis in solution A suggests that the relatively high K-content is an important factor in its toxicity; but the fact that solutions A/2 and A/10 also kill some animals, particularly those which have little ability to acclimate, indicates that K is not the only factor in toxicity. The non-toxic character of solutions B and B/2 and the slight toxicity of B/10 show that with a suitable calcium-potassium ratio the animals are able to live in a wide range of concentration. As a matter of fact, even concentrations of 3B/2 and 2B are non-lethal, with very few exceptions; and reconstitution will take place in both, though slowly.

None of the six solutions is rapidly lethal for *P. dorotocephala*. In solution A the animals die only after several days and in A/10 the animals live much longer than in the same volume of distilled water. In B/10 most individuals live for at least several weeks. It is evident that these solutions used as media for other agents cannot be primary factors in lethal effects occurring within a few hours after exposure, even though they may alter the susceptibility to another agent.

SUSCEPTIBILITY OF PLANARIA DOROTOCEPHALA TO KOH

An aqueous solution of KCN is alkaline in consequence of the reaction of KCN with water to form HCN and KOH. KCN m/1,000 in well water has a pH about 9.2. It has been shown in Table I that decrease in the alkalinity of a KCN solution decreases the susceptibility very greatly. This fact raises the question whether or to what extent the susceptibility of *Planaria* in the ordinary alkaline solution of KCN is due to the alkalinity rather than to KCN or HCN. To answer this question it is necessary to know something concerning

susceptibility to KOH. Data are given in Table III. Within a certain range of concentration, susceptibility to KOH depends on various factors besides concentration, viz., salt content of the water, the volume of solution in relation to number, size, and physiological condition of animals, and the frequency of renewal of the solution. In concentrations which are not lethal within 2 or 3 hours the gradual neutralization of the KOH by CO_2 produced by the animals decreases the pH so that a KOH solution which is slowly lethal in its

TABLE III

SUSCEPTIBILITY OF *Planaria dorotocephala* TO KOH IN DISTILLED WATER, WELL WATER, AND VARIOUS SALT SOLUTIONS, AS INDICATED BY TIME IN HOURS OF BEGINNING DISINTEGRATION OF 50 PER CENT OF HEADS

(Ten animals 18–20 mm. in length in each lot. The pH is 9.6 or higher in all except Lot 1, in which it is 9.2)

Lot No.	Solution	Time in Hours
1	KOH m/1,000 well water	All live
2	KOH m/500 well water	All live
3	KOH m/400 well water	All live
4	KOH m/300 well water	2
5	KOH m/1,000 solution A	15
6	KOH m/1,000 solution A/2	All live
7	KOH m/1,000 solution A/10	2
8	KOH m/1,000 solution B	All live
9	KOH m/1,000 solution B/2	All live
10	KOH m/1,000 solution B/10	2
11	KOH m/1,000 distilled H_2O	½

original concentration may be rendered non-lethal in a few hours by the worms if it is not renewed. In the series given in Table III the procedure was in all cases the same. Ten animals approximately 20 mm. in length were placed in 500 cc. of a given concentration of KOH by weight (not titrated). Since the purpose of these experiments was merely to determine the effect of certain differences in salt content on susceptibility to KOH rather than a study of variation in susceptibility with different methods of procedure, the solution was not renewed.

Lots 1–4 of Table III show that under these conditions m/1,000, m/500, and m/400 KOH in well water are not lethal, but that m/300 is rapidly lethal, the index time being 2 hours. Smaller, physiologi-

them to live indefinitely. The following examples will serve to illustrate the situation. Ten animals of 20 mm. size in 250 cc. distilled water without change gave an index time of 13 hours, in 125 cc. 24 hours, and with further decrease in volume or increase in number they live still longer. On the other hand, when the distilled water is frequently renewed, e.g., every hour, the survival time is only 6–8 hours for the large animals and still less for the small.

Murray (1928) has presented some data on the susceptibility of *Planaria* to salt solutions and has found that salt ratios approximating those of solution B (p. 91) (that is, high Ca and low K rather than the usual ratios of solution A) afford the most satisfactory salt medium for culture of the tissues of *P. dorotocephala*. In the experiments to be reported here, the salt ratios of the solutions A and B have been used in three concentrations each: the concentration as given (p. 91), and one-half and one-tenth of this concentration. With the object in view of determining how changes in Na, K, and Ca content and ratio alter susceptibility to KCN, the three concentrations of solutions A and B were used as follows. First, susceptibility of *P. dorotocephala* to these solutions brought to pH 7.5 by $NaHCO_3$ was determined. Second, since a KCN solution contains KOH, the effect of these solutions on susceptibility to KOH was determined. Third, susceptibility to KCN m/1,000 made up in these solutions was compared with that to KCN in well-water and distilled-water solutions. As regards the susceptibility to the salt solutions alone, it may be noted first that none of the six is rapidly lethal but that marked differences in susceptibility to them are associated with differences in physiological age and nutritive condition. In general, small, physiologically young animals (e.g., 7–8 mm. or less in length) are more susceptible to the higher concentrations and show a much greater ability to acclimate to salt solutions than the old, large animals (18–20 mm. or more in length). Young animals may be able to live indefinitely in a solution which kills the older after several days. For example, with animals from certain stocks which had been kept in the laboratory for some months all of the large animals gradually underwent complete disintegration in solution A/10, while all of the young animals in the same container remained intact. In solution A/2 laboratory stocks usually show a similar dif-

ference between old and young animals, though less marked, i.e., some of the young animals live and all of the old die, or more of the young than of the old live. This lower susceptibility of small animals in the lower concentrations of solution A is unquestionably a matter of acclimation, for the susceptibility of the small animals to sufficiently high concentrations is distinctly greater than that of the large. Animals of a given size from freshly collected stocks show a greater ability to acclimate to the salt solutions than those kept for long periods in the laboratory. In the very low concentrations susceptibility depends to some extent on volume and frequency of renewal of the solution in relation to the volume of tissue of animals. With such concentrations the susceptibility is less in a small volume of solution not renewed than in a large volume with frequent renewal.

In determining the effects of the different salt solutions, ten animals of the same size were washed once in the solution to be used and then brought into 250 cc. of the solution in a finger bowl, and the solution was not renewed. Solution A produces no visible effect at first; but in the course of a day or two the animals become incapable or almost incapable of progressive locomotion, although muscular contraction continues for days in the form of irregular bendings and contortions. In all lots observed thus far from various stocks in various condition, solution A has killed sooner or later, that is, after 4 days to a week or even more. In solution A/2 some of the old animals 16 mm. or more in length from old laboratory stocks die after 7–10 days, but animals of the same sizes from freshly collected stocks are less susceptible and often live for several weeks, probably indefinitely. Young animals, even from the laboratory stocks, are less susceptible than the old and in most cases live for several weeks, probably indefinitely. The occurrence of more or less acclimation of younger animals in solution A/10 has already been noted. All animals 18–20 mm. in length from old laboratory stocks die in 10–13 days, while animals 7–8 mm. in length from the same stocks and in the same container are intact and active after 16 days. It is possible that in such cases the death and disintegration of the large animals supplies additional salts and so makes continued life possible for the small animals; but this possibility does not alter the fact that the young animals remain intact and active while the old

begin to die after some days. Both large and small animals, newly collected, live, with occasional exceptions, for several weeks and probably indefinitely in solution A/10.

The solutions B and B/2 do not kill and are not toxic to *P. dorotocephala*, but B/10 may kill some of the larger animals of old stocks and occasionally a small animal in the course of 7–10 days, while the animals of new stocks remain intact at least for several weeks. Considering these susceptibilities in the light of Murray's work (Murray, 1928), it is evident that the calcium-potassium ratio in solution A is not suited to *P. dorotocephala*. The more or less complete paralysis in solution A suggests that the relatively high K-content is an important factor in its toxicity; but the fact that solutions A/2 and A/10 also kill some animals, particularly those which have little ability to acclimate, indicates that K is not the only factor in toxicity. The non-toxic character of solutions B and B/2 and the slight toxicity of B/10 show that with a suitable calcium-potassium ratio the animals are able to live in a wide range of concentration. As a matter of fact, even concentrations of 3B/2 and 2B are non-lethal, with very few exceptions; and reconstitution will take place in both, though slowly.

None of the six solutions is rapidly lethal for *P. dorotocephala*. In solution A the animals die only after several days and in A/10 the animals live much longer than in the same volume of distilled water. In B/10 most individuals live for at least several weeks. It is evident that these solutions used as media for other agents cannot be primary factors in lethal effects occurring within a few hours after exposure, even though they may alter the susceptibility to another agent.

SUSCEPTIBILITY OF PLANARIA DOROTOCEPHALA TO KOH

An aqueous solution of KCN is alkaline in consequence of the reaction of KCN with water to form HCN and KOH. KCN m/1,000 in well water has a pH about 9.2. It has been shown in Table I that decrease in the alkalinity of a KCN solution decreases the susceptibility very greatly. This fact raises the question whether or to what extent the susceptibility of *Planaria* in the ordinary alkaline solution of KCN is due to the alkalinity rather than to KCN or HCN. To answer this question it is necessary to know something concerning

susceptibility to KOH. Data are given in Table III. Within a certain range of concentration, susceptibility to KOH depends on various factors besides concentration, viz., salt content of the water, the volume of solution in relation to number, size, and physiological condition of animals, and the frequency of renewal of the solution. In concentrations which are not lethal within 2 or 3 hours the gradual neutralization of the KOH by CO_2 produced by the animals decreases the pH so that a KOH solution which is slowly lethal in its

TABLE III

SUSCEPTIBILITY OF *Planaria dorotocephala* TO KOH IN DISTILLED WATER, WELL WATER, AND VARIOUS SALT SOLUTIONS, AS INDICATED BY TIME IN HOURS OF BEGINNING DISINTEGRATION OF 50 PER CENT OF HEADS

(Ten animals 18–20 mm. in length in each lot. The pH is 9.6 or higher in all except Lot 1, in which it is 9.2)

Lot No.	Solution	Time in Hours
1	KOH m/1,000 well water	All live
2	KOH m/500 well water	All live
3	KOH m/400 well water	All live
4	KOH m/300 well water	2
5	KOH m/1,000 solution A	15
6	KOH m/1,000 solution A/2	All live
7	KOH m/1,000 solution A/10	2
8	KOH m/1,000 solution B	All live
9	KOH m/1,000 solution B/2	All live
10	KOH m/1,000 solution B/10	2
11	KOH m/1,000 distilled H_2O	½

original concentration may be rendered non-lethal in a few hours by the worms if it is not renewed. In the series given in Table III the procedure was in all cases the same. Ten animals approximately 20 mm. in length were placed in 500 cc. of a given concentration of KOH by weight (not titrated). Since the purpose of these experiments was merely to determine the effect of certain differences in salt content on susceptibility to KOH rather than a study of variation in susceptibility with different methods of procedure, the solution was not renewed.

Lots 1–4 of Table III show that under these conditions m/1,000, m/500, and m/400 KOH in well water are not lethal, but that m/300 is rapidly lethal, the index time being 2 hours. Smaller, physiologi-

cally younger animals show a higher susceptibility, and differences in nutritive condition also affect susceptibility. Lot 5, KOH m/1,000 in solution A, shows high susceptibility; but in both this and m/300 KOH in well water the K content is high enough to be lethal. In KOH m/1,000 in solution A/2 (Lot 6) all the animals live, and in KOH m/1,000 in solution B (Lot 8) and in solution B/2 (Lot 9) with low K and high Ca content all live; while to KOH m/1,000 in solution A/10 (Lot 7) and to KOH m/1,000 in solution B/10 (Lot 10) all are highly susceptible, the index time being 2 hours. The susceptibility is still higher to KOH m/1,000 in distilled water (Lot 11). The important point for present purposes is that KOH m/1,000 in well water and in certain concentrations of the salt solutions is entirely non-lethal (Lots 1, 6, 8, 9) but that with decrease in salt content it becomes lethal even when the salt content is sufficient to permit the animals to live for a long time or indefinitely (Lots 7, 10) and in distilled water is very rapidly lethal (Lot 11). It is evident from these facts that the KOH resulting from the decomposition of KCN in water is not the primary lethal factor in KCN m/1,000 in well water and in the higher salt concentrations. Even if the decomposition of KCN into KOH and HCN in such a solution is complete, the concentration of KOH will be m/1,000; and this is not lethal and, as a matter of fact, to all appearances not even toxic.

SUSCEPTIBILITY OF PLANARIA DOROTOCEPHALA TO KCN IN RELATION TO CERTAIN CHANGES IN SALT CONTENT AND pH OF THE SOLUTION

The question of susceptibility of *Planaria dorotocephala* to KCN in relation to salt balance and concentration of the solution and the effect of change in pH on this susceptibility present certain features of interest. Table IV gives data, all from 20 mm. animals of a single stock, on the susceptibility of *P. dorotocephala* to KCN m/1,000 in different concentrations of the salt solutions A and B. The experimental lots are in pairs, of which the first number of each pair (Lot 1 I, 2 I, etc.) gives the susceptibility in solutions in which the KOH resulting from decomposition of KCN is not neutralized and which are consequently alkaline with a pH of 9.6 or higher. The second number of each pair (Lots 1 II, 2 II, etc.) in each case gives the sus-

ceptibility in a solution of the same salt content and concentration as the first, but neutralized by HCl. In the first two pairs, alkaline and neutral solutions of KCN in solutions A and A/2, no marked differences in susceptibility appear. In both cases the susceptibility is

TABLE IV

SUSCEPTIBILITY OF *Planaria dorotocephala* TO KCN M/1,000 IN DISTILLED WATER AND VARIOUS SALT SOLUTIONS AT THE pH DETERMINED BY THE KCN AND WITH NEUTRALIZATION BY HCl. IN THE HIGHER SALT CONCENTRATIONS DISINTEGRATION WAS OBSERVED IN SAMPLES OF 5–6 ANIMALS RETURNED TO WELL WATER AT INTERVALS

(Animals 18–20 mm. in length)

Lot No.	KCN m/1,000 in--	pH	Time in Hours
1 I 1 II	Solution A	9.6+ 7	5 4
2 I 2 II	Solution A/2	9.6+ 7	6 4
3 I 3 II	Solution A/10	9.6+ 7	5 11
4 I 4 II 5 I 5 II	Solution B	9.6+ 7 9.6+ 7	10 6 7¼ 7
6 I 6 II	Solution B/2	9.6+ 7	6½ 6
7 I 7 II	Solution B/10	9.6+ 7	2 10½
8 I 8 II 9 I 9 II	Distilled water	9.6+ 7 9.6+ 7	½ 11 1 11

slightly higher in the neutral solutions (Lots 1 II, 2 II), but the slight differences are probably not significant. In Lots 3 I and 3 II, KCN in solution A/10, the susceptibility in alkaline solution is much higher than in neutral, the index time in the former being less than half that in the latter. Lots 4 I, 4 II, 5 I, 5 II, each pair alkaline and neutral in solution B; Lots 6 I and 6 II, alkaline and neutral in solution B/2; and Lots 7 I and 7 II, alkaline and neutral in solution

B/10, show that in B and B/2, as in A and A/2, neutralization does not decrease susceptibility, while in B/10, as in A/10, there is a great decrease in susceptibility with neutralization. In solutions B (Lots 4 and 5) and B/2 (Lot 6) neutralization apparently increases susceptibility as in A and A/2 (Lots 1 and 2), though this apparent increase may not be significant. The factor or factors concerned in determining the high susceptibility in the neutralized KCN-salt solutions have not been experimentally analyzed. Either the high K-content or the effect of the high salt concentration on ionization, or both, may be concerned.

Lots 8 and 9 are two pairs giving susceptibility in alkaline and neutral distilled water solutions of m/1,000 KCN. It is evident at once that in the alkaline solution (8 I, 9 I) the susceptibility is extremely high, being about the same as in KOH m/1,000 in distilled water (Lot 11, Table III). In the neutralized distilled-water solutions (8 II, 9 II) the susceptibility is much lower, the index time being 11 hours in both, that is, almost as low as in some neutral well water-KCN solutions (Lots 10 and 11, Table I).

It is an interesting fact that, not only in well water but in distilled water solutions of KCN, neutralization decreases the susceptibility very greatly. In the KCN–distilled-water solution the alkalinity is evidently the primary lethal factor rather than KCN or distilled water. In Lot 7, Table IV, KCN-B/10 the susceptibility is also much higher in alkaline than in neutral solution. That the hypotonicity is not the lethal factor in these solutions but merely increases the toxicity of KOH is shown by the following facts: First, in well water and balanced salt solutions of proper concentrations KOH is not toxic in a concentration as high as, or higher than, that in the KCN solutions (Table III). Second, in spite of the hypotonicity, susceptibility is greatly decreased by neutralization with HCl. With increase in osmotic pressure and approach to salt balance the toxicity of KOH decreases and KCN or HCN becomes the lethal factor but, except in the higher salt concentrations used, alkalinity increases susceptibility to cyanide. A comparison of Lots 1 I, 2 I, 3 I, 4 I, 5 I, 6 I, of Table IV with Lots 1–8 of Table I shows that susceptibility to KCN in all the salt solutions except B/10 (Lot 7 I) is somewhat lower than the average in KCN–well-water solutions. In the light of the facts

already considered, this difference is to be regarded merely as further evidence indicating the effect of salt concentration on the physiological effect of high pH.

SUSCEPTIBILITY OF PLANARIA MACULATA

Planaria maculata is not known to occur in the vicinity of Chicago; and even where it does occur, it cannot readily be obtained in as large numbers as can *Planaria dorotocephala*. The stock used in the following experiments was obtained from the vicinity of New Haven through the kindness of Dr. J. W. Buchanan. Because of the limited supply of material, smaller numbers of animals are used in each experiment; and the data given in Table V are less complete than those for *P. dorotocephala* but serve as a basis for comparison with that species as regards the more important points concerned.

Comparison of Lots 1 and 2 shows that susceptibility to KCN m/1,000 in well water is greatly decreased by neutralization as in *P. dorotocephala*. Susceptibility to KCN m/1,000 in solution A (Lot 3) is the same as in well water. In this lot susceptibility was determined as *P. dorotocephala*, by returning samples to water at intervals. Susceptibility to KCN m/1,000 in solution A/10 at pH 9.6+ (Lot 4) is higher than in well water, but with neutralization is greatly decreased (Lot 5). Lots 6–8 show the very high susceptibility to KCN m/1,000 in distilled water without neutralization, and Lot 9 shows a decrease of about 1,100 per cent in susceptibility with neutralization.

Unfortunately it was necessary to use for some of the experiments animals of smaller size which had undergone reconstitution and were therefore physiologically younger than the original stock. Lots 10–13 give data from such animals. Lot 10 shows that the susceptibility to KCN m/1,000 in well water at pH 9.2 is twice as high as in the older animals (Lot 1) but that, as in the older animals (cf. Lots 1 and 3), susceptibility in solution A is about the same as in well water (cf. Lots 10 and 11). In solution A/2, also at pH 9.6+, it remains the same (Lot 12) and is not altered by neutralization (Lot 13). As regards these susceptibility relations to alkaline and neutralized well water and to the three concentrations of solution A, *P. maculata* does not differ essentially from *P. dorotocephala* (see Tables I and

III). In both species susceptibility to KCN m/1,000 in well water is greatly decreased by neutralization, is not decreased at all by

TABLE V

SUSCEPTIBILITY OF *Planaria maculata* TO KCN M/1,000 IN WELL WATER, SALT SOLUTIONS, AND DISTILLED WATER AT pH DETERMINED BY THE KCN AND WITH NEUTRALIZATION BY HCl: SUSCEPTIBILITY TO KOH IN WELL WATER, SALT SOLUTIONS, AND DISTILLED WATER; SUSCEPTIBILITY TO DISTILLED WATER

(Lots 1–9 and 13–24 were 12–14 mm. in length; Lots 10–13 consisted of somewhat younger worms 10–12 mm. in length)

Lot No.	No. of Animals	Agent	pH	Time in Hours
1	10	KCN m/1,000 in well water	9.2	9
2	10	KCN m/1,000 in well water	7	38
3	10+	KCN m/1,000 in solution A	9.6+	9
4	5	KCN m/1,000 in solution A/10	9.6+	7
5	5	KCN m/1,000 in solution A/10	7	26
6	5	KCN m/1,000 in distilled water	9.6+	1
7	6	KCN m/1,000 in distilled water	9.6+	1¼
8	6	KCN m/1,000 in distilled water	9.6	¾
9	5	KCN m/1,000 in distilled water	7	11
10	6	KCN m/1,000 in well water	9.2	4½
11	6	KCN m/1,000 in solution A	7	4
12	6	KCN m/1,000 in solution A/2	9.6+	4
13	6	KCN m/1,000 in solution A/2	7	4
14	6	KOH m/1,000 in well water	9.2	All live
15	6	KOH m/500 in well water	9.6+	All live
16	6	KOH m/400 in well water	9.6+	36
17	6	KOH m/300 in well water	9.6+	1¼
18	5	KOH m/1,000 solution A	9.6+	2¾ (1 dies, 4 only heads)
19	5	KOH m/1,000 in solution A/2	9.6+	All live (2 heads disintegrate)
20	5	KOH m/1,000 in solution A/10	9.6+	2½ (All live, 4 heads disintegrate)
21	6	KOH m/1,000 in distilled water	9.6+	¾
22	6	Distilled water	6.6	96
23	6	Distilled water	6.6	98 (2 live)
24	6	Distilled water	6.6	102 (3 live)

neutralization of KCN m/1,000 in solutions A and A/2, but is again greatly decreased by neutralization of KCN in solution A/10 and in distilled water. This point was discussed in the preceding section.

The third section of Table V gives susceptibility of *P. maculata* to KOH in well water, in solutions A, A/2, A/10, and in distilled water. In KOH m/1,000 and KOH m/500 in well water (Lots 14,

15) with the same procedure as for *P. dorotocephala* all animals live indefinitely; but KOH m/400 in well water (Lot 16) is lethal, the index time being 36 hours; and in KOH m/300 in well water the index time is 1½ hours. According to these results, *P. maculata* 12–14 mm. in length is somewhat more susceptible to KOH than *P. dorotocephala* 20 mm. in length, for the latter lives indefinitely in m/400 and the index time is 2 hours in m/300 (Table III, Lots 3 and 4).

In KOH m/1,000 in solution A (Lot 18, Table V) most heads disintegrate, but the bodies remain alive and later even reconstitute heads. *Planaria dorotocephala*, on the other hand, dies in this solution, the index time being 15 hours (Lot 5, Table V). As already noted, this solution is out of balance for *P. dorotocephala*, and the K concentration is high enough to be toxic. In KOH m/1,000 in solution A/2 (Lot 19, Table V) two heads disintegrate; otherwise all animals remain alive. *Planaria dorotocephala* also lives in this solution (Lot 6, Table III). In KOH m/1,000 in solution A/10 (Lot 20, Table V) all live except for disintegration of four heads, but in this solution *P. dorotocephala* has an index time of only 2 hours and all die. With the method of procedure employed the disintegration of heads only results from the fact that the solutions concerned are rather rapidly lethal for the most susceptible regions; but before they kill the less susceptible, more posterior levels, the pH is altered by the CO_2 production of the animals, so that the solution is no longer lethal.

Lot 21 shows a susceptibility to KOH m/1,000 in distilled water of three quarters of an hour, approximately the same as that of *P. dorotocephala* (Lot 11, Table III, ½ hour). Lots 22–24 (Table V) show susceptibility in distilled water, each lot consisting of six animals in 250 cc. distilled water without change. In spite of the fact that each lot consists of six instead of ten animals, the susceptibility of P. *maculata* to distilled water is much lower than that of *P. dorotocephala*, the index time of *P. maculata* varying from 96 to 102 hours in the three lots, with two or three of the animals remaining intact for at least a week, as compared with 13 hours for *P. dorotocephala* and no animals surviving. This difference in susceptibility in the two species is striking. Evidently a very low salt content is adequate for *P. maculata*. If the animals live 2 or 3 days in distilled water,

most of them are likely to live longer, apparently because they add salts to the medium. Also, if some of the animals of a lot die within 2 or 3 days in distilled water, the others are more likely to remain alive. Apparently the death and disintegration of the animals increase the salt content of the water.

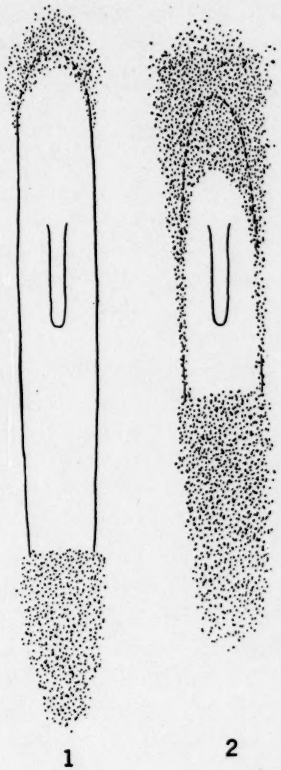
In general, *P. maculata*, from the stock and of the size used in most of the experiments, is somewhat less susceptible to KCN, much less susceptible to distilled water, and perhaps slightly more susceptible to KOH than *P. dorotocephala*. In KCN m/1,000 in distilled water, both alkaline and neutral, the susceptibility of the two species is essentially the same (cf. Lots 8 I, 8 II, 9 I, 9 II, Table IV, with Lots 6–9, Table V). Evidently for *P. maculata*, as for *P. dorotocephala*, alkalinity increases susceptibility to KCN and becomes the primary lethal factor in the KCN–distilled-water solution.

As regards salt solutions, no data are tabulated for *P. maculata*, but a few experimental results are briefly stated. Solution A is toxic for *P. maculata;* the animals gradually become more or less incapable of co-ordinated locomotion, and after several days some individuals die. In this solution, however, a considerable degree of acclimation apparently occurs, particularly in the smaller animals, which often show more or less recovery of locomotor activity after several days in the solution. The cut ends of pieces usually remain open in this solution, and pieces die more frequently than whole animals. In solution 4A/5 some deaths may occur, but acclimation is frequent and in pieces more or less reconstitution occurs after acclimation, but is retarded. In solutions 2A/3 and A/2 intact animals live and pieces undergo reconstitution in the latter concentration about as well as in water. Solution B and even solution 2B are not appreciably toxic for *P. maculata*. It should perhaps be stated that this is true for stocks of *P. maculata* which have lived for several weeks in the well water used in this laboratory and which has a high Ca-content. Whether it would be true for *P. maculata* living in a water of low Ca-content or low osmotic pressure has not yet been determined.

AXIAL DIFFERENTIAL SUSCEPTIBILITY AS AFFECTED BY THE EXPERIMENTAL CONDITIONS

Differential susceptibility to KCN m/1,000, pH 9.2, in well water. —In KCN m/1,000 in a natural water without neutralization the

longitudinal differential in susceptibility is essentially similar for the two species. It has been recorded for *Planaria dorotocephala* in an earlier paper (Child, 1913) and is only briefly reviewed here. The margins of the head and the posterior growing-tip of the body are the first regions to show disintegration of the ectoderm and body wall, and complete disintegration of the head may occur while other parts are still more or less motile. From the head region disintegration progresses posteriorly to the level of fission, the lateral margins being more susceptible than median regions at a given level, and in *P. dorotocephala* the dorsal more susceptible than the ventral body wall. Figure 1 shows an early stage; Figure 2 a more advanced stage of disintegration in *P. dorotocephala* under these conditions.[1] In *P. maculata* under similar conditions the ventral body wall is apparently more susceptible than the dorsal. The region posterior to the level of fission, that is, the posterior zooid region, is more susceptible than the region immediately anterior to this level, so that disinte-



FIGS. 1 AND 2.—An early and a later stage of disintegration of *Planaria dorotocephala* in KCN m/1,000 in well water at pH 9.2. Lateral margins and dorsal surface are more susceptible than median and ventral regions of the body wall. The anterior boundary of intact ventral body wall is indicated by a broken line. When two posterior zooids are present, the more posterior is more susceptible (Fig. 1) and the whole posterior zooid region is more susceptible than the posterior part of the anterior zooid (Fig. 2).

[1] These and following figures illustrating differential susceptibility are diagrammatic representations of stages in the course of disintegration of ectoderm and body wall. The disintegrated portions are merely indicated by shading. No attempt is made to show conditions in the alimentary tract. The boundary between intact and disintegrating tissue is relatively sharp, and the course of disintegration along the body is definite, as the figures indicate.

gration of the body wall is complete in the posterior earlier than in the anterior zooid region (Fig. 2). In large individuals of *P. dorotocephala* with a long posterior zooid region two, sometimes three, zones of different susceptibility may be distinguished in this region, particularly in lower concentrations of KCN in which disintegration is less rapid and regional differences appear more clearly (Child, 1913). These regions apparently represent different, physiologically determined zooids, and the experimental results on fission in *P. dorotocephala* agree with the data on susceptibility in indicating that in large animals two, or even three, posterior zooids may be present (Child, 1910). In such cases the most posterior of the zooid regions is most susceptible, and each region is somewhat more susceptible than that next anterior to it. In the material of *P. maculata* available, only one, or in a few large individuals perhaps two, posterior zooids have been distinguishable by their susceptibility.

3

FIG. 3.—A stage of disintegration of *Planaria dorotocephala* in KCN m/1,000 in well water at pH 6.5. The median ventral region is slightly more susceptible than lateral and dorsal regions. The posterior zooid region is relatively somewhat less susceptible than in alkaline KCN.

The susceptibility of the alimentary tract depends on nutritive condition. In animals which have been heavily fed, the alimentary tract often appears to be more susceptible to alkaline KCN and to KOH than the body wall and bursts through it, producing a wound which becomes a region of early disintegration. This occurs more frequently in or near the middle regions than near the ends and suggests that the susceptibility of the alimentary tract is greatest near the pharynx. In animals which have been starved for several weeks, susceptibility of the alimentary tract is much lower than that of the body wall and other tissues, so that

the alimentary tract often remains intact or almost intact for a time after other tissues are disintegrated. In both *P. dorotocephala* and *P. maculata* large animals are less susceptible than small, but both large and small show essentially the same longitudinal and lateromedian differential in the anterior zooid, i.e., to a certain postoral level; but in the small animals only one posterior zone of susceptibility appears; and in animals removed from egg capsules just before hatching, differential susceptibility indicates merely a posterior growing-region, most susceptible at its posterior end.

The effect on differential susceptibility to KCN of decrease in pH.— As Table I indicates, the susceptibility to KCN decreases with decrease in pH to pH 6.5 and increases with further decrease in pH. Incidentally it may be noted that pH 6.5 is well above the pH at which HCl alone becomes lethal, viz., pH 5.4 (Table II). The longitudinal differential in susceptibility decreasing from the head to the level of fission persists at all pH's used, but the rate of progress of disintegration decreases with decrease in pH to 6.5. With decrease in pH to about 6.5 the relatively high susceptibility of the lateral margins of the body disappears, and the median ventral region becomes slightly more susceptible than the lateral and dorsal (Fig. 3). With further decrease in pH the margins again become more susceptible, and the dorsal more susceptible than the ventral body wall.

In KCN solutions with pH below 6, disintegration is not closely associated with death if the animals remain in the solution. When returned to water, however, dead or dying parts disintegrate while other parts recover. In this way it can be determined that the longitudinal differential in susceptibility is essentially the same in these as in alkaline KCN solutions. Moreover, it is found that the parts which disintegrate on return to water appear thinner and somewhat different in color from other parts while in the solution. After recognition of these changes the progress of death can be followed more or less closely in the solution.

In slightly acid KCN the susceptibility of the posterior zooid region is often decreased to a somewhat greater extent than that of the anterior zooid, but different individuals differ in this respect, apparently with difference in degree of physiological isolation and stage of development of the posterior zooid or zooids. With increase in

acidity of the KCN solution the relative susceptibility of the posterior zooid region increases again.

The first section of Table V shows that susceptibility of *P. maculata* to KCN in well-water, hypotonic salt solution, and distilled water is greatly decreased by neutralization but the susceptibility of this species in acid KCN has not yet been determined.

The changes in susceptibility to KCN in *P. maculata* show the same general course as in *P. dorotocephala* but differ in certain details. For example, in alkaline KCN pH 9.2 the lateral margins are the most susceptible regions at any level, but the ventral surface is usually more susceptible than the dorsal. With a still higher pH, however, the dorsal surface becomes more susceptible than the ventral, i.e., the higher susceptibility of the dorsal surface appears at a higher pH than in *P. dorotocephala*. In neutral and slightly acid KCN solutions susceptibility decreases laterally and dorsally from the median ventral region, and the lateral margins do not show high susceptibility. A few experiments with more acid KCN solutions (not tabulated) show that with increase in acidity the high susceptibility of the lateral margins and the dorsal surface reappears as it does in *P. dorotocephala*.

Differential susceptibility to HCl at different pH.—As Table II indicates for *P. dorotocephala*, HCl solutions in well water with pH 5.4 or higher are either not lethal to all individuals or kill only the head regions. It should also be noted that to these solutions the small, physiologically younger animals are less susceptible than the larger (compare the two sections of Table II). This fact has been repeatedly confirmed by students in the laboratory and is interpreted as indicating that the smaller younger animals possess greater capacity than the larger ones for acclimating or acquiring tolerance to this pH range. With further decrease in pH the small animals become more susceptible than the large. Even in those concentrations which are only partly lethal, differential susceptibility appears in the disintegration of heads or in the usual course of death along the axes of some individuals.

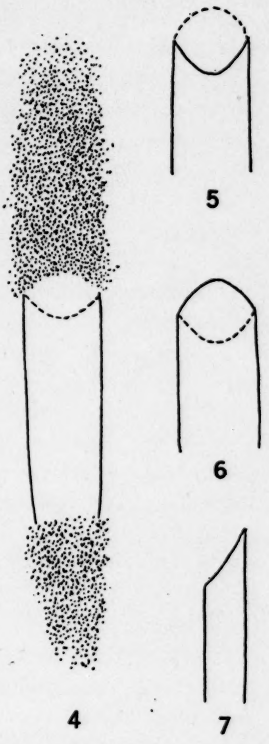
In general the longitudinal differential in susceptibility to HCl from the head to the level of fission is similar to that in KCN and other agents. Death and disintegration begin at the head and pro-

gress posteriorly. In the lower lethal concentrations of HCl the region of the posterior zooids is usually relatively less susceptible than in alkaline KCN. In long animals with two posterior zooids the more posterior of the two disintegrates about the same time as the anterior zooid (Fig. 4), while the more anterior may remain intact after all other parts are disintegrated. These differences suggest that the posterior zooids possess a slightly greater ability to acclimate to a certain range of pH than the anterior; and if they are physiologically younger regions, as many facts indicate, this is to be expected.

In these lower lethal concentrations, e.g., about 5.4–5.0, the median ventral region is distinctly more susceptible than lateral and dorsal regions at any given level, so that disintegration progresses posteriorly with the median ventral region in advance. This differential is indicated in Figure 5, ventral, Figure 6, dorsal, Figure 7, lateral aspect of a disintegrating animal.

With decrease in pH below 5 susceptibility increases rapidly (Table II), and the relative susceptibility of the posterior zooid region also increases. The longitudinal differential persists and maintains the same direction, but the lateral margins become more susceptible.

At about pH 4 or somewhat lower the acidity becomes irritating, producing violent contortions and much slime secretion, chiefly from the lateral margins



FIGS. 4–7.—Differential susceptibility in HCl, pH 5.4–5.0. Figure 4, a stage of disintegration showing that median ventral regions are more susceptible than lateral and dorsal and that the susceptibility of the posterior zooid region is decreased as compared with that in alkaline KCN. Figure 5, ventral, Figure 6, dorsal, Figure 7, lateral view of anterior boundary of intact body wall in a disintegrating animal to show the differential between median ventral, lateral, and dorsal regions.

and the dorsal surface. With the appearance of the irritating effect the susceptibility is greatly increased; the posterior zooids again become visible as regions of high susceptibility; the susceptibility of the lateral margins becomes higher than that of median regions, as in alkaline solutions; and finally the dorsal surface becomes more susceptible than the ventral, except in the head region where the ventral surface apparently remains more susceptible. In these acid concentrations the differentials appear much more clearly in animals returned to water after different lengths of time in the acid than in animals remaining in the acid. The increase in general susceptibility and in relative susceptibility of lateral margins and dorsal surface occurs at lower acid concentrations in the anterior zooids of small animals than in large animals, and in the posterior zooids usually at a lower concentration than the anterior. These differences are evidently associated with the fact that the anterior zooids of small animals and the posterior zooids of large animals are physiologically younger than the anterior zooids of large animals.

A few experiments with *P. maculata* at low pH give essentially similar results. In more or less alkaline, neutral, and slightly acid KCN, susceptibility decreases in *P. maculata* from the median ventral region laterally and dorsally. In acid with a pH low enough to kill slowly without irritation this differential also appears; but as the pH becomes low enough to irritate, slime secretion is greatly increased and the lateral margins and dorsal surface become more susceptible than the median ventral region.

There can be little doubt that this change in the mediolateral and dorsoventral differential with decrease in pH below a certain point is associated with the irritating action of the acid on the marginal slime glands and perhaps on other structures of the dorsal surface. Apparently the dorsal surface is more sensitive than the ventral to this irritation, for in the contortions of the animals the ventral surface is as fully exposed as the dorsal.

Differential susceptibility in KOH.—In lethal concentrations the longitudinal differential in both *P. dorotocephala* and *P. maculata* appears as in the other agents used, disintegration beginning with the head and progressing posteriorly to the fission level. The posterior zooids also disintegrate early, as in alkaline KCN. In *P. doroto-*

cephala lateral margins and dorsal surface at any given level disintegrate before median and ventral regions. In well-water–KOH solutions the whole picture is very similar to that in alkaline KCN (Fig. 1). In KOH in the higher concentrations of neutral salt solutions used, the difference between margin and median and between dorsal and ventral regions is least marked, and increases with the increase in susceptibility accompanying decrease in neutral salt concentration. In all toxic and lethal KOH solutions the slime secretion is increased, the slime glands of the lateral margins showing the most conspicuous effect in this respect; but the dorsal surface is also involved.

In *P. maculata* the margins are the most susceptible regions at a given level to KOH, but the differential is less marked than in *P. dorotocephala*. In concentrations which kill very slowly, the margins may be no more susceptible than median regions; but in higher concentrations, the higher relative marginal susceptibility appears.

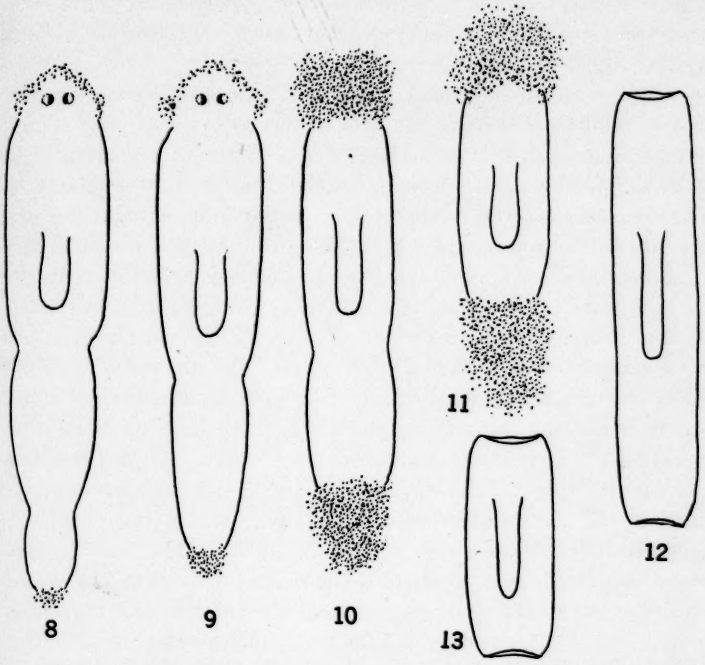
As regards the dorsoventral differential also, the two species differ somewhat as regards the pH range at which change occurs. In *P. dorotocephala* the dorsal body wall disintegrates before the ventral in KCN solutions above pH 8 and in lethal KOH solutions, although with decrease in concentration of KOH and approach to the upper limit of acclimation the differential between dorsal and ventral decreases. In *P. maculata* the ventral body wall is more susceptible than the dorsal in alkaline KCN (pH 9.2), and in the lower killing concentrations of KOH; but experiments with higher alkalinities (not tabulated) show beyond question that, as alkalinity increases, the dorsal body wall becomes more susceptible than the ventral. Apparently there is some difference between the two body surfaces in both species, such that with increase in alkalinity the dorsal, and with decrease in alkalinity the ventral, surface becomes relatively more susceptible; but the pH range within which these changes occur apparently extends farther on the alkaline side for *P. maculata* than for *P. dorotocephala*. In KOH disintegration is often complicated by extrusion of the pharynx through the body wall. In some cases also parts of the alimentary tract break through the dorsal body wall, apparently as the result of swelling. The injuries thus produced become regions of high susceptibility and show early disintegration.

Differential susceptibility in distilled water.—As in other agents, disintegration begins at the head and progresses posteriorly in the first zooid, the lateral margins disintegrating slightly in advance of median regions. The susceptibility of the posterior zooids is relatively low in distilled water, and they usually remain intact until after the anterior zooid is disintegrated. Apparently they are able to develop a higher degree of tolerance for distilled water than the anterior zooid.

Differential susceptibility to KCN m/1,000 in neutral salt solutions.—In KCN m/1,000 made up in salt solutions A, A/2, B, or B/2 disintegration in general is not closely associated with death. Slight disintegration usually occurs on the margins of the head and at the extreme posterior end (posterior growing-region); but the animal may retain its general form for a day or more, though obviously dead, and finally undergo a slow maceration. This does not mean that differential susceptibility is obliterated in these solutions. Observation of the animals in the solutions indicates clearly that differential susceptibility is in general very similar to that in alkaline KCN in well water. The visible changes consist in a decrease in thickness of the body, a change in color to grayish, and more or less bending of the body so that the ventral surface is concave transversely and the dorsal surface concave longitudinally, at least in the anterior region, the parts thus bent remaining relatively rigid for some hours but finally becoming limp. These changes begin at the head and in the posterior zooid region. The head becomes bent posteriorly over the dorsal surface, and from the head the change progresses posteriorly to the fission plane, margins in advance of median region, though the bending is less marked in the middle region than at the ends.

In *P. dorotocephala* the posterior zooid region of the longer animals usually shows two zones of distinctly different susceptibility with definite boundaries (Fig. 8). Since in animals of this length the posterior fission-piece usually undergoes a second fission soon after the first, and since the levels at which such fissions occur coincide approximately with the boundaries of the zones of different susceptibility, these zones are regarded as representing the posterior zooids present. Where two such zones are present in the posterior zooid re-

gion, the posterior zone is always more susceptible than the anterior, as indicated by the fact that it becomes thinner with margins bent ventrally earlier than the anterior zone. The apparently greater de-



FIGS. 8–13.—Differential susceptibility of *Planaria dorotocephala*, as indicated by disintegration and recovery on return to water after different periods of exposure to KCN m/1,000 made up in hypertonic balanced salt solutions. Figure 8, disintegration in water after short exposure; head and posterior tip disintegrate. The presence of two posterior zooids is indicated by constrictions and ventral inturning of lateral margins at certain levels. Figure 9, an individual with one posterior zooid indicated after short exposure. Figures 10 and 11, disintegration in water after longer exposure. Figures 12 and 13, recovery and beginning reconstitution in water of the less susceptible regions of the two individuals represented in Figures 10 and 11.

gree of constriction at the anterior end of each zone results from the greater degree of turning ventrally and inward of the margins in this region. This differential suggests that the anterior end of each of these zones is more susceptible than its other levels.

Some individuals show only a single zone of differential susceptibility in the posterior zooid region (Fig. 9). These appear to be cases in which a third zooid has not yet become sufficiently distinct to appear as a definite zone. In *P. maculata* only one distinct zone of differential susceptibility has been observed in the posterior zooid region.

Differential susceptibility to KCN in salt solutions is even more clearly demonstrated by returning animals to water after various periods of exposure. By exposing twenty-five or fifty animals of the same size to the solution at a given time, and then at regular intervals returning a certain number (e.g., five or ten animals) to water, the differential appears with great clearness. In fact, the differences in susceptibility are even more sharply marked than in well-water solutions of KCN m/1,000. On return to water the parts which are dead or beyond the possibility of recovery disintegrate rapidly, usually within an hour or less in well water and still more rapidly in water of lower salt content; while other parts remain intact, recover, and later develop the missing parts. Figures 10 and 11 show disintegration after return to water from KCN m/1,000 in solution B, and Figures 12 and 13 show the recovery of the less susceptible regions of the same individuals. In this way it can be shown with diagrammatic clearness that susceptibility decreases from the head posteriorly in the first zooid, that the posterior zooid region is more susceptible than the posterior region of the anterior zooid, and that in large individuals two zones, the posterior more susceptible than the anterior, are usually distinguishable in it. Lateral margins are somewhat more susceptible than median regions, and in KCN in the higher salt concentrations this differential is not affected by neutralization, but in KCN in solutions A/10 and B/10 it disappears with neutralization as it does in KCN–well-water solutions and the median ventral region becomes most susceptible.

A comparison of the parts in which the visible changes described above have occurred in the solution with the parts of the same individuals which disintegrate after return to water shows that the two represent the same body regions and are approximately identical in extent. In short, the differentials in susceptibility are not obliterated in KCN made up in more or less nearly isotonic or hyper-

tonic salt solutions but, on the other hand, are very distinct, even though death and disintegration are not closely related as they are under various other conditions.

In the higher salt concentrations protrusion of the pharynx through the body wall sometimes occurs, making a wound which becomes a region of high susceptibility and early disintegration. Such pharyngeal protrusion occurs in various agents but is absent in various others, and therefore must be regarded as a specific effect of certain agents on the highly specialized pharynx.

DISCUSSION

The differences in index time.—Taking KCN m/1,000 in well water as a convenient starting-point, we find that the susceptibility of *Planaria* decreases with decrease in pH of this solution to about ph 6.5 and with further decrease of pH increases again. In HCl–well-water solutions the susceptibility of *P. dorotocephala* increases with increase in pH below 5.4. Evidently a hydrogen-ion concentration which is not in itself lethal increases the toxicity of the acid KCN solution, probably by setting free HCN.

The KCN m/1,000–well water solution is alkaline because of the formation of KOH, the pH being approximately that of KOH m/1,000 in well water. The alkalinity is not, however, the factor which kills in the KCN solution, for KOH in well water does not kill until its concentration is more than double that in the KCN solution. It may be noted that the decrease in susceptibility with decrease in pH parallels in direction the change in rate of oxygen consumption (Hyman, 1925; Anderson, 1927), but it does not necessarily follow that the decrease in respiration is itself the factor determining decrease in susceptibility. The increase in susceptibility to KCN with decrease in pH below 6.5 obviously results directly or indirectly from the increasing hydrogen-ion concentration.

The changes in susceptibility to KCN with changes in neutral salt content of the solution present certain points of interest.

In the higher salt concentrations A, A/2, B, B/2, susceptibility to KCN is somewhat lower than in well water, but is not further decreased, in fact, it is apparently increased by neutralization. In solution A/10 without neutralization it is about the same as in the

higher salt concentrations but is greatly decreased by neutralization. In solution B/10, the lowest salt concentration used, the susceptibility in alkaline solution is considerably higher than that in the other salt solutions and is greatly decreased by neutralization (Table IV).

Potassium hydroxide m/1,000 does not kill in solutions over a wide range of neutral salt concentration; but in solutions A/10 and B/10 (Table III) it kills all animals, the index time being 2 hours; and in distilled-water solutions the susceptibility is still higher ($\frac{1}{2}$ hour). To KCN m/1,000 in distilled water the susceptibility of both species is about the same as to KOH m/1,000 in distilled water, and is greatly decreased by neutralization (Tables III, IV, V). Apparently in the KCN–distilled-water solution it is the KOH, not the KCN or HCl, which limits the survival time. Obviously, it is not primarily the hypotonicity, for this is but little affected by neutralization, while the susceptibility is greatly decreased. The hypotonicity merely serves to increase the susceptibility to KOH or to high pH.

In a paper read before the American Society of Zoölogists in 1925, J. W. Wilson (1925) maintains that

> the disintegration of *P. maculata* in m/1000 KCN in pond water is due to the cytolytic effect of the hypotonic medium which invades the body locally at the eosinophilic slime-producing region along the margin. This region is the most susceptible part of the body surface and its rupture permits invasion. . . . . It is therefore concluded that the disintegration of *P. maculata* in m/1000 KCN in pond water is not initiated by the specific effect of the KCN on the oxidative metabolism of the worm as a whole, but is produced by the cytolytic action of the hypotonic medium before the KCN has had time to produce its specific effect.

Wilson's statement reads as if he believed that the hypotonicity, not the KCN, were responsible for the rupture of the margins. This obviously cannot be the case, for the animals live in pond water; but in KCN m/1,000 in pond water the margins disintegrate first. This would seem to be due to some sort of action of the KCN solution rather than the hypotonicity.

Wilson has failed to take into account the alkalinity of the KCN solution and its effect on susceptibility. The data presented above show that the alkalinity rather than the hypotonicity is the chief factor in determining the degree of susceptibility. But KOH in the

concentration present in the KCN solution does not kill except in highly hypotonic solution; therefore the only conclusion possible in the light of all the facts seems to be that in the KCN–well-water solution the KOH increases the susceptibility to the KCN or HCN and that death is due to the latter, not to the KOH.

If the pond water used by Wilson had a very low salt concentration, the KOH, instead of the KCN, may have been the chief lethal factor, for hypotonicity increases susceptibility to KOH. This seems probable in the light of his statement that the susceptibility to KCN is much lower in "approximately isotonic" Ringer's solution than in pond-water solution. In my own experiments the various modified Ringer's solutions do not greatly decrease the susceptibility to KCN below that of well-water solutions; but in the lowest salt concentration, B/10, it is considerably higher than in well water, and in KCN–distilled-water solution it is still higher. The hypotonicity of the solution is undoubtedly a factor in the physical changes which the cells undergo before, during, or after death; but it is not the factor which kills them, even in KCN–distilled-water solution. If it were, we should expect death and disintegration instead of healing and growth of new tissue to occur on a cut surface of *Planaria* in water.

Acclimation.—In the course of the experiments some incidental observations on the occurrence of acclimation or acquirement of tolerance have been made. Acclimation is most clearly evident with HCl between pH 5.8 and 5 (Table II). At pH 5.8 all the small animals live and remain intact, while three (30 per cent) of the large animals lose their heads. At pH 5.6 six (60 per cent) of the small animals remain intact, four die, and three (30 per cent) of the large animals lose their heads. At pH 5.4 six small animals and only one of the large animals live and remain intact. At pH 5 the small animals are less susceptible than the large, the index time for the small being 12 hours and for the large, 5 hours. At pH 4.6 the susceptibility of large and small varies somewhat in different lots, but averages about the same. At still lower pH the small animals are more susceptible than the large. Evidently the smaller, physiologically younger animals are able to acclimate, to a greater degree than the larger older animals, to a certain range of pH (5.8–5.0). These observations confirm the work of J. W. MacArthur (1920), and further

confirmation has been repeatedly obtained in the laboratory work of students. Indications of some degree of acclimation to the salt solutions A and B have also been observed, particularly in the reconstitution of pieces; but these will be considered elsewhere.

The data on susceptibility to KOH give no evidence of acclimation, but the experiments performed were not adapted to show acclimation if it occurs. Anderson (1927) found no evidence for the occurrence of acclimation to alkali. In certain concentrations of KOH which are primarily lethal, the animals lower the pH through their own CO_2 production, so that animals or parts not killed within the first few hours remain alive, or the death and disintegration of some individuals decreases the pH sufficiently so that others are able to live. This, however, is not acclimation but alteration of the medium by the animals.

Differential susceptibility.—It is important to note first of all that in both species the longitudinal differential in susceptibility from the head to the level of fission, i.e., within the anterior zooid, appears in the body wall and is constant in direction under all lethal conditions considered in the present paper, except in a few cases of differential acclimation in which it is reversed. Early disintegration of the body wall in the pharyngeal region in consequence of protrusion of the pharynx, or of swelling and bursting of the alimentary tract, is obviously an incidental result of injury to these regions.

The growing region at the extreme posterior end is also a region of high susceptibility. The region of the posterior zooid or zooids is in general more susceptible than the posterior portion of the anterior zooid, and the presence of two posterior zooids is indicated by two distinct zones differing in susceptibility; the posterior of the two zones is in general the more susceptible.

Under certain conditions, however, the relative susceptibility of the posterior, as compared with the anterior, zooid region is somewhat altered. In the lower lethal concentrations of HCl the relative susceptibility of the posterior zooid region is somewhat decreased, so that it is often the last part of the body to disintegrate. This suggests that this region has a slightly greater ability than the anterior zooid to acclimate to a certain range of pH. Such a difference is similar to that between young and old animals; and since various

facts already on record indicate that the posterior zooid differs to some extent from the anterior, as young animals differ from old, it is to be expected. With further decrease in pH the relative susceptibility of the posterior zooid region again increases.

In neutral and slightly acid KCN solutions the relative susceptibility of the posterior zooid region also shows a decrease; that is, this pH range decreases greatly the susceptibility to KCN of all parts of the body but decreases that of the posterior zooid region somewhat more than that of other parts. Since there is no indication of acclimation to KCN m/1,000, this is probably to be regarded as indication that the primary effect of the low pH on the physiologically younger posterior zooid region is greater than on other parts. Young animals are primarily more susceptible than old to low pH, even when the low pH does not kill and acclimation occurs later. The greater primary susceptibility of the young animals is indicated by a greater decrease in motor activity at first, but within the range of acclimation the motor activity of the young animals increases later more rapidly and to a greater degree than that of the old. And finally, in distilled water the relative susceptibility of the posterior zooid region is also decreased. The distilled water used was neutral or very slightly acid, and it has not been determined as yet whether the decrease in relative susceptibility of the posterior zooid region is an effect of the pH or whether it represents a slight degree of acclimation to the hypotonic medium.

The differential in susceptibility between lateral margins and median region and between dorsal and ventral surfaces does not remain constant under the different conditions of experiment. In KCN solutions over a certain range of pH around neutrality the susceptibility of the body wall in the transverse plane decreases laterally and dorsally from the median ventral region. Both above and below this pH range the lateral margins become more susceptible than the median region; and at about the same, or still higher and lower pH, the dorsal surface becomes more susceptible than the ventral. In KCN made up in the higher concentrations of the salt solutions without neutralization, the lateral margins and dorsal surface are somewhat more susceptible than median and ventral regions, but the difference is not as great as in alkaline KCN–well-water solutions. In KCN-

A/10, KCN-B/10, and KCN–distilled-water solutions the susceptibility of the margins is much higher than that of the median region. This differential disappears with neutralization in well-water solutions, i.e., the high susceptibility of lateral margins and dorsal surface disappears and susceptibility decreases laterally and dorsally from the ventral and median region.

As regards the general significance of the data on differential susceptibility discussed here, attention is called briefly to certain points.

First, we can scarcely escape the conclusion that the high degree of constancy in direction of the longitudinal differential or gradient of susceptibility in the body wall of the anterior zooid under the conditions considered in this paper and under many others observed must have a physiological basis of some sort. Moreover, it is difficult to conceive that basis in any other than quantitative terms, since the differential in susceptibility shows the same direction for external agents widely different in character. But, even if a longitudinal quantitative differential does exist in the body wall and perhaps in the parenchyma, it does not follow that the same differential is present in the alimentary tract. So far as the data on susceptibility give any evidence on this point, they suggest that the pharyngeal region and the middle portions are the most susceptible regions of the alimentary tract, the susceptibility decreasing both anteriorly and posteriorly.

Second, the occurrence of a differential in susceptibility in the transverse plane which is not constant for all conditions observed, but differs in certain definite ways under different conditions, raises the question of the physiological basis of such differences. In such forms as *Planaria* the mid-ventral region of the body wall precedes lateral and dorsal regions in development and is apparently primarily the most active region of the body wall in the transverse plane. On the basis of these facts it is suggested that the transverse differential with susceptibility decreasing laterally and dorsally from the mid-ventral region represents the general primitive physiological differential in the absence of specific effects of particular agents on particular highly specialized organs. If this be true, the appearance under certain conditions of the high marginal and dorsal suscepti-

bility in both species may be interpreted as a more or less specific effect of particular conditions on certain specialized parts. There can be little doubt that the high marginal susceptibility represents exactly that. Along the lateral regions are large numbers of slime glands. Both high pH and low pH which give high marginal susceptibility excite these glands to increased secretion, as is readily seen. Apparently they are more sensitive to such excitation than other regions of the body at the same level and so under the exciting conditions become more susceptible. J. W. Wilson (1925) has recognized the high susceptibility of these marginal slime-gland cells in alkaline KCN but has not examined them in neutral and acid solutions.

The high susceptibility of the dorsal surface appears under conditions similar to, but in some cases more extreme than, those giving high marginal susceptibility and probably depends on essentially similar factors. Dorsal and ventral surfaces are markedly different in appearance and structure, and unquestionably possess a considerable difference in function. The changes in relative susceptibilities of dorsal and ventral surfaces under different conditions must be in some way associated with the differences in specialization which they have undergone during development. In other words, the mediolateral and the dorsoventral differentials in susceptibility, which change in definite ways with certain changes in conditions, must indicate specific physiological differences in the parts concerned.

In the original description of differential susceptibility to alkaline KCN in *Planaria* (Child, 1913) it was suggested that the high marginal susceptibility might be due to the presence of sensory cells along the margin and the high dorsal susceptibility to the lesser thickness of the dorsal as compared with the ventral body wall. The interpretation suggested above, of differential excitation of differently specialized regions, is based on much more extensive evidence as regards *Planaria* and is in agreement with many data of differential susceptibility in other forms. It may be that sensory cells contribute to the high susceptibility of both margin and dorsal surface, but apparently the slime cells are the most important factors in marginal susceptibility, and they may play a part in determining dorsal susceptibility. But whatever the particular factors involved, the only possible conclusion seems to be that the changes in the medio-latero-

dorsal differential under different conditions indicate specific physiological differences in the different regions concerned.

Differences in susceptibility in the two species.—The differences in susceptibility between *P. dorotocephala* and *P. maculata* are slight. Comparing the large animals, that is, *P. maculata* 12–14 mm. and *P. dorotocephala* 20 mm. in length, the former is less susceptible than the latter to KCN m/1,000 in well water; but the differences in KCN m/1,000 in salt solution and in distilled water are slight. The large animals of the stocks of *P. maculata* used for experiment were slightly more susceptible to high pH than *P. dorotocephala*, but those were not as near the maximum size of *P. maculata* as the 20 mm. individuals of *P. dorotocephala*, so that the difference is not significant. *Planaria maculata* is much less susceptible than *P. dorotocephala* to distilled water and to the hypertonic salt solutions used; that is, it will live in a wider range of osmotic pressure and salt ratio than *P. dorotocephala*. Concerning the physiological basis of these specific differences in susceptibility, no definite conclusions can be drawn, but it seems probable that the difference in susceptibility to extremes of osmotic pressure and range of salt ratio is associated with the difference in habitat of the species. *Planaria dorotocephala* occurs, so far as is known, only in spring water with a relatively high calcium content; while *P. maculata* is found much more widely, occurring in ponds and streams in which the Ca-content and often the salt content in general are much lower.

The general significance of differential susceptibility.—Susceptibility to KCN in well water, in hypotonic salt solutions, and in distilled water decreases with decrease in pH to a certain point and then increases again. Oxygen consumption decreases with decrease in pH (Hyman, 1925; Anderson, 1927), but we have no evidence that it increases with still further decrease in pH. In the higher salt concentrations, decrease in pH to neutrality does not decrease susceptibility, but whether it has any effect on respiration is not known. Susceptibility to KOH increases with increase in hypotonicity, but even distilled water does not appreciably increase respiration.

Undoubtedly, innumerable changes in external conditions may alter susceptibility to agents added to the changed environment, and the changes in susceptibility may or may not parallel the quantita-

tive changes in metabolism. These and other similar facts do not conflict in the slightest degree with the significance of differences in susceptibility as indicators of difference in physiological condition or activity. The point of importance is that, with a particular given environment or complex of conditions as a starting-point, the individual and regional differences in susceptibility in an organism which are not specific for particular agents when these are added to the given environment, but are similar in direction for different agents, indicate non-specific or quantitative differences in physiological condition existing in that particular environment.

In another environment the differences in physiological condition and in the susceptibilities may both be altered, but again in this environment the evidence indicates that the differences in susceptibility which are not specific for particular agents added to the environment must be indicators of quantitative differences in physiological condition. It is perhaps needless to add that we cannot attribute any definite significance to differences in susceptibility between two different species, except as indicators of differences in the two protoplasms, until we know much more about the nature of the protoplasms and of the action of external agents on them.

With respect to KCN, it makes no difference as regards the general conclusion whether susceptibility to KCN depends directly upon its power to inhibit oxidation or on some other action or actions on protoplasm. Whatever the nature of its action or that of any other agent on a protoplasm, the evidence indicates that the non-specific differences in susceptibility must indicate non-specific or quantitative physiological differences.

In earlier discussions of differential susceptibility it has been stated that such susceptibility appears to be, in a general way, an indicator of the rate of metabolism or, more specifically, of oxidative metabolism as indicated by respiratory exchange. In certain cases and by means of certain oxidation-reduction reactions a parallelism between differential susceptibility and respiratory rate has been demonstrated or indicated, and in other cases indirect evidence for the existence of such parallelism has been presented (Child, 1924, chap. vii; 1928). It has become increasingly evident, however, with the progress of investigation on differential susceptibility and in

other fields that the chief significance of differential susceptibility does not depend merely on this parallelism. In the less highly specialized protoplasms, under normal aërobic conditions the parallelism between differences in susceptibility and differences in respiratory rate apparently exists in many cases, if not generally; and the rate of respiration appears to be more or less closely correlated with the other essential factors which determine the level of physiological activity. In certain cells, e.g., tumor cells and embryonic cells, under anaërobic conditions glycolysis rather than respiration may be a more adequate measure of the level of physiological activity (Warburg and Others, 1926). Moreover, it is a well-known fact that inhibition of embryonic development may be brought about by certain agents without appreciable decrease, and in some cases with increase, of respiration for a certain length of time (e.g., Warburg, 1910). Evidently the agents which produce such effects act primarily on other factors than the oxidations, or increase these while inhibiting certain other activities. In other words, they alter the protoplasmic system; but if their action is sufficient in degree, life does not continue indefinitely and the oxidations are sooner or later inhibited. Moreover, the evidence at hand indicates a general parallelism between susceptibility to such agents (e.g., NH_4OH, NaCl) and respiratory rate. After the protoplasmic system has been altered by the action of such agents, the differential susceptibility of the altered system to other agents may parallel some other physiological differential than respiratory rate. At present we know practically nothing concerning the effects of such alterations of the system by a certain agent or agents on the differential susceptibility of the altered system to other agents; but that such alteration may change the relation previously existing between differential susceptibility and a particular factor of the system, e.g., respiration, is of course possible. Granting this, it appears difficult to escape the conclusion that even under such conditions differences in susceptibility which are not specific for particular agents are still indicators of quantitative physiological differences.

If we regard a living protoplasm as a system continuously undergoing dynamic equilibration, it seems evident that that protoplasm in which the changes constituting equilibration are proceeding most

rapidly must be most susceptible to extreme disturbance by an external factor whatever the character of the disturbance, and at the same time able to acclimate or equilibrate most rapidly to relatively slight disturbances. At present such relation between equilibration and disturbance appears to provide a basis for interpretation of the data of differential susceptibility. Under normal aërobic conditions the oxidations appear to be essential factors in the equilibration process, at least in most protoplasms; and so far as this is the case, a general parallelism between differences in susceptibility and differences in respiration may be expected; but it does not follow that such parallelism will be found in all protoplasms or under all conditions.

At present the only other way of accounting for the facts of differential susceptibility which suggests itself is to assume that all agents employed in concentrations or intensities above the limit of tolerance act in essentially the same way on a protoplasmic system. For example, we may suppose that all of them bring about surface changes which have the same effect as regards the life of the system and that differential susceptibility depends on differences in condition of the surface. Unless we assume, however, that all agents bring about these changes in the same way—and this appears scarcely possible—this interpretation becomes essentially the same as the others; i.e., any sufficient disturbance of surface conditions leads to death, and the susceptibilities of surfaces will differ with quantitative differences in their physiological condition. Moreover, if conditions at the surface are as important for metabolism as is usually believed, we should expect to find a relation between susceptibility dependent on surface action and the metabolism of the cell or region concerned.

In conclusion, the fact may be emphasized once more that differences in susceptibility exist which are specific for particular agents as well as those which are not specific. The marginal slime cells of *Planaria* exhibit such specific susceptibilities, and in the higher animals and man we find all degrees of specificity in the relation between particular organs and particular agents. It is evident that these specificities result from the specialization of organs and parts. We find them appearing in the course of embryonic development and of

evolution; but as we turn back to the earlier stages of development and the simpler organisms, we find that these specific susceptibilities become less conspicuous and disappear, and that only the general differential susceptibility which is not specific for different agents is present. At present we can find no other basis for this differential than a quantitative physiological differential in the protoplasm concerned, and many lines of evidence support the view that such a differential represents a physiological axis in its simplest, most general terms.

SUMMARY

1. Susceptibility of *Planaria dorotocephala* to KCN m/1,000 in well water decreases greatly with decrease in pH to about 6.5, and with further decrease in pH increases again.

2. In HCl in well water above pH 5.4 more or less acclimation occurs, the smaller, physiologically younger animals showing a greater degree of acclimation than the larger, older. Below pH 5.4 susceptibility increases with decrease of pH.

3. The susceptibility of *P. dorotocephala* to KOH differs very greatly with osmotic pressure of the medium. In well water, concentrations below m/300 do not kill. In markedly hypotonic solutions and in distilled water, KOH m/1,000 kills rapidly; while this concentration in salt solutions which are nearly isotonic or hypertonic is not toxic.

4. The data on susceptibility to KOH and KCN show that KOH is not the primary lethal factor in KCN m/1,000, except in distilled water or highly hypotonic media; but the great decrease in susceptibility resulting from the neutralization of KCN m/1,000 shows that the alkalinity of the KCN solution increases the susceptibility to KCN.

5. Susceptibility to distilled water depends on number and size of individuals in relation to volume of water and frequency of change. Apparently salts are given off by the animals; and if volume of water is not too large or change too frequent in relation to number of individuals, life may continue indefinitely.

6. The higher concentrations of the balanced salt solutions used, although undoubtedly hypertonic, kill very slowly; and lower concentrations over a wide range are not toxic. Susceptibility to KCN

m/1,000 made up in the salt solutions is slightly lower in the higher salt concentrations than in well water and is not decreased by neutralization; but with decreasing salt concentration, susceptibility to KCN is increased by alkalinity and greatly decreased by neutralization.

7. The changes in general susceptibility in *P. maculata* with change in pH and osmotic pressure are similar to those in *P. dorotocephala*. *Planaria maculata* is less susceptible to distilled water and is able to live in a wider range of salt concentration and ratio than *P. dorotocephala*. These differences are probably associated with differences in the habitat of the two species.

8. The longitudinal differential in susceptibility in the body wall of the first zooid is the same in direction under all conditions considered in the present paper, but the rate of progress of death from the head posteriorly may differ widely under different conditions.

9. In *P. dorotocephala* the differences in susceptibility in the posterior zooid region often indicate two, and occasionally three, zooids. Under certain conditions, e.g., in the lower acid concentrations, relative susceptibility of the posterior zooids is decreased, apparently because they acclimate to a greater degree than the anterior zooid. With increase in acidity the relative susceptibility of the posterior zooid region increases. In distilled water also, decrease in relative susceptibility of the posterior zooids occurs, which may represent some degree of acclimation to the hypotonicity.

10. Differential susceptibility in the transverse plane differs with different conditions. In KCN–well-water solutions over a certain pH range which differs somewhat for the two species, and in HCl within certain limits of pH, susceptibility decreases laterally and dorsally from the median ventral region. With increase of pH above or decrease below a certain range, the alkalinity or acidity becomes a stimulating or irritating factor. Under these conditions the lateral margins become the most susceptible regions in the transverse plane, and the dorsal surface becomes more susceptible than the ventral. This change in susceptibility is accompanied by increased slime secretion of the marginal slime glands, and also to some extent of the dorsal surface.

11. In KCN-salt solutions without neutralization lateral margins

and dorsal surface are more susceptible than median and ventral. In the higher salt concentrations the difference is slight, but in KCN-A/10, KCN-B/10, and KCN–distilled-water solutions the margins are much more susceptible than median regions. With neutralization this differential disappears.

12. The differences in susceptibility in the longitudinal axis which are similar in direction for a certain range of concentration or degree of action of all agents used are interpreted as indicating a quantitative or non-specific physiological differential, while the differentials in the transverse plane which change in direction under different conditions are considered to indicate the existence of specific physiological differences in the parts concerned.

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THE CORPUS LUTEUM HORMONE. II. METHODS OF EXTRACTION[1]

FREDERICK L. HISAW, H. L. FEVOLD, AND R. K. MEYER
Department of Zoölogy, University of Wisconsin

INTRODUCTION

IN PREVIOUS papers (Weichert, 1928; Hisaw, Meyer, and Weichert, 1928; Hisaw, 1927 and 1929) several physiological properties of extracts prepared from the corpora lutea of the sow were described, and a few of the chemical characteristics were also briefly given (Hisaw, 1929). This paper reports in some detail the chemical procedure used in the preparation of active extracts as well as certain chemical properties of the substance, in so far as they have been determined.

Extracts of lutein tissue have been prepared for physiological work by various investigators. In general these extracts have been of two kinds, saline and lipoidal. Concordant results have, however, not been obtained by their use, and, in fact, the results in many cases have been distinctly contradictory. Pearl and Surface (1914) inhibited ovulation in hens by means of saline extracts and suspensions of dried fat-free lutein tissue. Corner and Hurni (1918), using similar extracts, failed to inhibit the ovulation of rats; while Loeb (1917) failed to obtain positive results in the guinea pig. Kennedy (1925), however, inhibited ovulation in rabbits by the use of saline extracts of dried and fresh corpus luteum tissue. Papanicolaou (1926) was able to inhibit ovulation in guinea pigs by means of a lipoidal fraction of corpus luteum tissue, while Johnston and Gould (1926) could not obtain a lipoidal extract which would inhibit the action of the follicular hormone. Loewe and Lange (1927) found only slight indications of delayed oestrum after injection of fat-soluble extracts of corpora lutea in amounts representing 36 grams of fresh tissue per mouse. Parkes and Bellerby (1927) prepared an extract

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by which they inhibited the oestrus cycle of mice. Their preparation was also lipoidal in nature, consisting of the acetone-soluble fraction of the ether extract of the tissue. The active principle was labile, being destroyed by heat, exposure to air, or on being left in ether solution. Contrary to this, Payne, Van Peenan, and Cartland (1928) found the active substance to be stable. They prolonged the oestrus cycle of the guinea pig 4–9 days by injection of the unsaponifiable fraction of the corpus luteum. Gley (1928) succeeded in inhibiting ovulation of rats by an extract of corpora lutea which was prepared by extracting the tissue with water acidulated with tartaric acid. Macht, Stickels, and Sechinger (1929) prepared an ethyl acetate extract of corpus luteum tissue which inhibited the cycle of the guinea pig. The corpus luteum tissue was exhaustively extracted with ether and petroleum ether, and then extracted with ethyl acetate. Corner and Allen (1929) produced progrestational proliferation in castrated rabbits and survival of the embryos by the use of lipoid extracts of corpora lutea.

From this brief review of the literature it can readily be seen that there is little or no consistency in the results obtained by the various investigators. This may be due, in part, to the use of different animals in the experimental work. However, the fact that similar results have been obtained with extractions of corpus luteum tissue representing entirely different fractions would seem to indicate that the method of assaying the extracts is at fault. Every investigator, with two exceptions, as far as known, has used inhibition of ovulation as the criterion of activity. That the corpora lutea normally inhibit ovulation seems well established; but the fact that extracts of tissues other than the corpus luteum as well as certain vital dyes, also do this, seems to throw doubt on the value of this reaction as a valid test. Facts to substantiate this statement will be presented in another paper.

One of the chief tests used for the extracts described in the present paper was the relaxation of the pelvic ligaments of the guinea pig. The purified extracts have, however, been tested by various reactions ordinarily attributed to the corpus luteum, such as inhibition of ovulation, vacuolation of the vaginal mucosa in castrated rats, and the production of progestational proliferation of the uterus

of castrated rabbits. These results are to appear in other papers, but it may be mentioned that they have been positive in every case.

EXPERIMENTAL METHODS

The experimental results will be presented in the following order: (I) extraction of fresh tissue, (II) purification of the extract, (III) physical and chemical properties of the substance.

I. EXTRACTION OF FRESH TISSUE

The corpora lutea used for extraction were dissected from fresh sow's ovaries, which had previously been selected for their high corpora lutea content. The lutein tissue was ground in a meat grinder and finely pulped with sand in a mortar. In the initial investigation a number of solvents were used on the fresh tissue in attempts to obtain the active material in solution. Of these, ether, acetone, petroleum ether, neutral ethyl alcohol, and benzol failed to extract the active principle as determined by the relaxative test (Hisaw, 1929). Occasionally a slight positive text was obtained from the ether and neutral-alcohol extracts; but these results were neither constant nor strongly positive, so the use of these solvents was abandoned. Saline extracts of the fresh tissue gave positive results in every case. The saline extracts, however, deteriorated rapidly because of putrefaction, and great care had to be exercised in their use.

Ninety-five per cent ethyl alcohol, which had been acidified with hydrochloric acid (2 cc. concentrated HCl per 98 cc. alcohol), extracted the relaxative substance better than any solvent used; hence it was employed in all subsequent work. The finely minced tissue was extracted twice at room temperature, allowing 48 hours for each extraction. A third extraction was found to give only a slight increase in the yield, so usually was not made. The volume of acid alcohol in cubic centimeters, used in each case, was equal to twice the weight of the tissue in grams. After the active principle has been extracted from the tissue by means of acid alcohol, it is then soluble in neutral alcohol, and can be purified quite readily and prepared for injection.

Before proceeding to the details of the purification of the extract described above, further experiments on the extraction of the tissue should be related. They are of no importance as far as the prepara-

tion of usable extracts is concerned, but they throw some light on the possible nature of the active principle. The action of pepsin on the lutein tissue, for example, does not destroy the relaxative hormone but renders it soluble in water and alcohol, so that it can then be extracted from the tissue by either of these two solvents. Pancreatin, on the other hand, completely destroys the hormone. After treatment of the fresh tissue with pancreatic solution it was not possible to get a positive reaction from any subsequent extractions. If the fresh tissue is treated with acidulated water, the active substance is thereby rendered soluble in neutral alcohol. In this procedure the tissue was first treated for 48 hours with acidulated water, the acid was neutralized, and the water evaporated without being removed from the tissue. The tissue was then extracted with neutral alcohol, and the extract prepared for injection. The results from such extracts are always strongly positive. It is true, as previously mentioned, that the relaxative substance cannot be extracted from fresh corpora lutea by neutral ethyl alcohol or acetone, but it can be obtained from the tissue residue, which has been treated with these two solvents through the use of acid alcohol. The acid-alcohol extract in this case seems to be comparable in potency for the relaxation reaction to a similar extract of whole fresh tissue; namely, 0.1 cc. equal to 1.00 grams of fresh tissue is usually sufficient to relax the pelvic ligaments of a virgin guinea pig while in full oestrum.

II. PURIFICATION OF THE ACID-ALCOHOL EXTRACT

The acid-alcohol extract of the tissue is removed from the residue by straining through cheesecloth in a press. It is then diluted with one-third its volume of water. Material, insoluble in this concentration of water and alcohol, gradually separates out and is filtered off. The precipitate is completely inactive and can therefore be discarded. It consists chiefly of lipoids, a considerable portion of which are phospholipins.

The solution is neutralized to a pH of 6.8 or 7.0, whereupon a flocculent precipitate settles out. This consists chiefly of protein and degradation products of proteins together with some fatty material. As first precipitated, this precipitate shows slight activity. This is completely lost if the material is redissolved in acid alcohol and reprecipitated as before. The precipitate after the second precipitation is discarded, and the second extract added to the first.

After neutralization the solution is evaporated to semidryness. This can be done in vacuo at 40° C., although some trouble is experienced because of foaming. It is therefore more convenient to evaporate the solution in an air-dryer at 35°–40° C. The residue is repeatedly extracted with alcohol, which has been dried over lime, until the last portions of alcohol show only a faint yellow color. The active substance is soluble in the alcohol and is thereby freed from the salt of neutralization as well as other material which remains insoluble in the alcohol.

The alcohol extract is evaporated to semidryness as before, and residue extracted repeatedly with acetone. The acetone extract ordinarily contains small amounts of the active substance. This is removed from solution by evaporating this fraction to dryness and re-extracting with acetone. The united residues from the acetone extractions are emulsified in water. A small amount of calcium or sodium chloride is added, and the phosphatides precipitated by the addition of an equal volume of acetone. The active principle is soluble in the aqueous acetone solution; while the phospholipins can be removed by centrifuging, and discarded.

After being freed from the phosphatides, the solution is evaporated to semidryness, as before, and again extracted with dried alcohol, using 100 cc. portions until no more is taken up in solution. This alcoholic solution contains the active principles in concentrated form and is prepared for injection. This is done by adding water to the alcoholic solution and evaporating in the air-dryer to remove the alcohol. The resulting aqueous solution of the active substance is a clear brownish liquid and can be concentrated to any desired strength. On standing in the icebox for a few days, small amounts of material settle out. The solution is filtered through a Berkefelt filter, thereby freeing it from any insoluble material and bacterial contamination. An amount of this preparation equivalent to 1 gram of fresh tissue is usually sufficient to relax the pelvic ligaments of a guinea pig in oestrum.

III. CERTAIN PHYSICAL AND CHEMICAL PROPERTIES OF THE SUBSTANCE

The active principle responsible for the relaxation reaction is quite unstable. It is completely destroyed by boiling the aqueous solutions for short periods of time. It is also completely killed by

heating the dried material above 50° C. for 48 hours. If left at room temperature in dry form, the activity gradually disappears. It is stable in fairly strong acid solutions, but concentrated acids injure it. In alkaline solutions the substance is destroyed; especially is this so of fairly concentrated alkali. The substance is stable in neutral alcohol or aqueous solution. Such solutions have been kept for 6 or 7 months in the icebox with no detectable decrease in activity.

The substance, after extraction with acid, is soluble in water and in alcohol, or in mixtures of the two in any proportion. It is not as readily soluble in so-called "absolute alcohol" as in alcohol containing small amounts of water. It is readily absorbed by foreign material from alcoholic solution, and it is often difficult to free the alcohol-insoluble material from the active principle due to this fact. It is insoluble in all of the organic solvents, such as ether, acetone, petroleum ether, cold benzol, ethyl acetate, and butyl alcohol. If fat is present, these solvents will sometimes take up small amounts of the active material; but by evaporating the solution and re-extracting the residue, the active material can be separated from the fat.

The substance passes readily through a Berkefelt filter. Sterile aqueous solutions can therefore be prepared. It also passes through ultra-filters and dialyzes rather slowly through collodion bags which have previously been tested with Congo red. It is interesting to note that the relaxative hormone, as it exists in the blood, does not pass through ultra-filters, nor does it dialyze through collodion bags to any extent. This can be readily explained on the basis that the hormone in the blood is combined with the pseudoglobulins, while in the extract it is presumably in the free condition due to the mild hydrolysis of the acid alcohol.

The material in the extract contains nitrogen but no phosphorous or sulphur. Slight tests for phosphorous are often obtained; but if the material is repeatedly purified, no phosphorous is present. It gives none of the protein tests except the ninhydrin reaction, indicating that the extract is practically free from degradation products of proteins. It is free from all fatty substances which are extractible by means of ether, acetone, or petroleum ether.

DISCUSSION

Before entering into a discussion of the procedures just presented, it should be made clear that we are dealing with an extract and not a purified substance. It should also be mentioned that the chief test employed has been the relaxation of the pelvic ligaments of the guinea pig, and the properties given are with regard to this reaction. Although, in addition to relaxing the pelvic ligaments of the guinea pig, this extract does inhibit the oestrous cycle, vacuolate the mucous membrane of the rat vagina, produce the progestational proliferation of the uterus in castrated rabbits, and quiets the contractions of the turtle uterus, we do not wish to claim that all these reactions are due to one and the same substance. As a matter of fact, we feel there are good reasons to think the contrary. That is, this extract seems to contain more than one active substance. A further fractionation of the preparation described here lends weight to the idea that there may be more than one active substance obtained from the corpus luteum by our method. By this process, in fact, we have been able to obtain a crystalline fraction which relaxes the pelvic ligaments of the guinea pig and a second fraction which, although it does not cause such relaxation, does inhibit oestrum and vacuolate the vaginal mucosa. What other reactions these fractions are capable of giving are not known; they await further study which we hope to report in subsequent papers. The details of the preparation of the crystalline fraction will also be reported soon. It may be mentioned, however, that less than 0.5 mg. of the crystalline material is sufficient to relax the pelvic ligaments of a guinea pig while in full oestrum.

A very small amount of follicular hormone is present in the extract even though the material has been repeatedly extracted with solvents which should remove it. This amount of follicular hormone, however, is not capable of materially influencing such reactions as the relaxation of the pelvic ligaments or progestational proliferation of the castrate rabbit's uterus but, when present in larger amounts, does interfere with inhibition of oestrum and vacuolation of the vaginal mucosa of rats.

The corpus luteum extracts used in the relaxation work reported in the first paper of this series (Hisaw, 1929) were prepared similar to the method described here. These extracts were capable of pro-

ducing the several physiological reactions mentioned here, and the probability of there being a single corpus luteum hormone responsible was discussed. At that time methods of further fractionation of these preparations had not been discovered; and this knowledge, as presented here, throws doubt on the earlier assumption.

It has been stated that the active material in our extract is insoluble in all fat solvents such as ethyl ether, acetone, or petroleum ether. This is true only when little or no fatty material is present, since the presence of fats seems to have a definite effect on the solubility of the active material in these solvents. When the crude product is extracted with acetone, the acetone solution contains a small amount of active material, which can be removed from solution by evaporation and re-extraction. The residue, which then contains all the active material, is entirely insoluble in all fat solvents, showing that the small amount which is taken up in the acetone is not due to any solubility in the solvent but is due to the presence of fatty materials.

It would seem that the hormone or hormones elaborated by the corpora lutea must be different chemically from the hormone obtained from the follicles. While it is impossible to say a great deal about the solubility of a substance before obtaining it in a pure form, still we do know that the material in our extracts is not lipoidal in nature, as evidenced by its insolubility in all lipoidal solvents, whereas the follicular hormone is soluble in these solvents. This fact would seem to be of importance since we have here a method by which it is possible to separate the active principles of the corpora lutea from that of the follicles. It is, of course, true that the corpus luteum tissue contains some follicular hormone which will always be present in an extract unless removed by some specific method. The final product obtained by this method, while in a semidry condition, can be extracted with acetone, ether, or cold benzol without materially decreasing the potency of the residue. This, it would seem, should remove all the follicular hormone which may be present. Having the corpus luteum extracts free from follicular hormone provides better opportunity to study the relationships in physiological reactions.

The relaxative substance seems to be associated with the proteins in the tissue. It has been shown that the hormone appears in the blood of certain animals during pregnancy. Here it is associated

with the pseudoglobulins and cannot be extracted from semidried serum with neutral solvents. After hydrolysis of the semidried serum with acid, however, the hormone becomes soluble in water and neutral alcohol. The same holds true in the tissues. The hormone cannot be extracted from tissues by means of any pure neutral solvent. However, after hydrolysis by means of acid or pepsin it becomes soluble in alcohol and in water. It can be extracted from tissue with normal saline solution, which would also seem to point to the probability that the active principle is associated with a protein fraction and that hydrolytic agents free the hormone from the material to which it is attached.

The relaxative substance, while not species specific, seems to be present in the blood during pregnancy in different amounts for different species, and apparently may be entirely lacking in certain forms. An example of an animal in which this hormone seems to be absent is the cow. We have never been able to produce an unquestionably positive relaxative reaction from extracts of the blood during pregnancy, placenta, or corpora lutea of this animal. We have no explanation for this interesting exception, although it does add weight to our ideas concerning the probability of the presence of more than one hormone in the sow corpus luteum. The corpus luteum of the cow, though it does not yield the relaxative substance, does function as the sow corpus luteum in other physiological reactions, as far as studied. Whether or not it is true that there is more than one hormone secreted by the corpora lutea remains for further work to determine; and in the meantime we may, for convenience, refer to the relaxative substance as *a* corpus luteum hormone but not *the* corpus luteum hormone.

SUMMARY

1. A method is given for the preparation of a fat- and protein-free extract containing the substance or substances necessary to produce certain physiological reactions ordinarily attributed to the corpus luteum.

2. Evidence is presented relative to the probability of there being more than one hormone secreted by the corpus luteum of the sow.

3. The physical and chemical properties of the substance, as far as they have been determined, are given.

4. The method given permits the separation of the follicular hormone and the active substances of the corpus luteum.

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